BIOCHEMISTRY AND MOLECULAR BIOLOGY OF BINDING PROTEINS FOR PLANT GROWTH REGULATORS

A thesis submitted for the degree of Doctor of Philosophy of

De Montfort University, Leicester

by

Yun-Heng Zhang

The Norman Borlaug Institute for Plant Science Research,
De Montfort University, Leicester

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Abstract

Plant growth regulators have a vital role in plant growth and development. The cellular response to these regulators depends on the presence and the action of specific receptors. The plant growth regulators and their receptors act together in complexes which determine the final effects of the plant growth regulators. In the research reported here, emphasis has been given to the regulation of the activity of the receptors themselves. The regulation of the N-1-naphthylphthalamic acid (NPA) receptor through phosphorylation and dephosphorylation and the regulation of the auxin binding protein (ABP) through gene manipulation have been investigated.

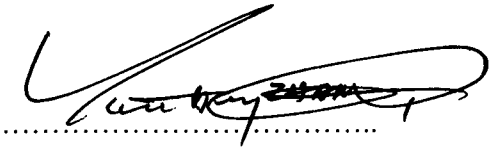
NPA, an auxin transport inhibitor, was found to bind specifically to a crude membrane preparation from sugar beet seedling leaf cell suspension cultures. The *in vitro* binding was optimal at pH 4.5 and 4°C. Binding parameters for NPA binding were determined by Scatchard analysis. The dissociation constant (Kd) and binding protein concentration were found to be 1.71 x 10^{-7} mol dm^{-3} and 220 pmoles g^{-1} membrane protein respectively. It was found that the amount of specific ^{3}H-NPA binding was significantly increased by adding Mg^{2+}ATP to the binding assay solution; treatment of membrane preparations with acid phosphatase, prior to the NPA binding assay, resulted in lower specific binding. ATP activation and phosphatase inactivation were culture stage dependent. Although a considerable effect could be detected when using cells from day 8 (representing the linear phase), the same treatment did not alter the binding if cells from day 1 (representing lag phase) or day 14 (representing the stationary phase) were used. These observations have strongly highlighted the possible involvement of a phosphorylation and dephosphorylation mechanism *in vivo* in the regulation of the activity of the NPA receptor. High phosphatase activity was found in the supernatant, but not in the membrane pellet, after 50,000 *g* centrifugation.

The presence of a membrane-bound auxin receptor, ABP, was demonstrated by Scatchard analysis in sugar beet seedlings. The Kd value and the receptor concentration were found to be 2.15 x 10^{-6} mol dm^{-3} and 68 pmoles g^{-1} membrane protein. The protein could be solubilised either with the detergent Triton X-100 or by acetone-washing, with a recovery of about 40%. An acetone-solubilised ABP preparation could be partially purified by DEAE-Sephacel ion exchange chromatography, NAA-linked AH-Sepharose 4B affinity chromatography or Sephadex G-200 gel filtration. The recovery after any of these chromatographic treatments was very low so that successive chromatography for further purification was unsuccessful. The low level of detectable binding after purification resulted mainly from the low abundance of ABP in the plant material. Non-radioactive labelling and detection techniques were used to show that an ABP-probe hybridized to sugar beet genomic DNA during dot blotting.

The present study has indicated that receptor activity could be regulated by a phosphorylation and dephosphorylation mechanism in plants. The investigation has also suggested that the effect of plant growth regulators on plant development could be regulated through the manipulation of the expression of their receptor genes.

Declaration

This thesis is entirely my own work and at no time has been submitted for another degree.



Yun-Heng Zhang

I certify this statement is correct.

M C Elliott

M. C. Elliott

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Abbreviations

A: absorbance

ABA: abscisic acid

ABP: auxin binding protein

AH: aminohexyl

AP: alkaline phosphatase

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

bp: base pair

cDNA: complementary DNA

4-Cl-IAA: 4-chloro-indole-3-acetic acid

CTP: cytidine triphosphate

2,4-D: 2,4-dichlorophenoxyacetic acid

dATP: deoxyadenosine triphosphate

dCTP: deoxycytidine triphosphate

DEAE: diethylaminoethyl

dGTP: deoxyguanosine triphosphate

DIG: digoxigenin

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

dpm: disintegrations per minute

DR: 2,4-D requiring

DTT: dithiothreitol

dTTP: deoxythymidine triphosphate

dUTP: deoxyuridine triphosphate

EDC: *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDTA: ethylenediaminetetraacetic acid

ER: endoplasmic reticulum

FPLC: fast protein liquid chromatography

GTP: guanosine triphosphate

HPLC: high pressure (performance) liquid chromatography

IAA: indole-3-acetic acid

IBA: indole-3-butyric acid

IgG: immunoglobulin G

K_{+1}: rate constant of association

K_{-1}: rate constant of dissociation

Ka: association constant

Kd: dissociation constant

KD: kilodalton

KDEL: Lys-Asp-Glu-Leu

mAb: monoclonal antibody

mRNA: messenger ribonucleic acid

NAA: napthylacetic acid

NBT: nitroblue tetrazolium

NDR: no 2,4-D requiring

NPA: N-1-naphthylphthalamic acid

NTP: nucleotide triphosphate

PAA: phenylacetic acid

PAGE: polyacrylamide gel electrophoresis

PCV: packed cell volume

PGR: plant growth regulator

pNPP: p-nitrophenylphosphate

PVPP: polyvinylpolypyrrolidone

RNA: ribonucleic acid

RNase: ribonuclease

rpm: revolutions per minute

sABP: soluble auxin binding protein

SDS: sodium dodecyl-sulphate

T-DNA: Transforming DNA of the Ti-plasmid

Ti plasmid: tumour inducing plasmid

TIBA: 2,3,5-triiodobenzoic acid

Tris: tris(hydroxymethyl)aminomethane

TTP: thymidine triphosphate

UTP: uridine triphosphate

UV: ultraviolet

Content

Chapter 5

Chapter 1 General Introduction

Unlike other eukaryotic organisms, plants must meet the specific demands of a sessile, autotrophic habit. It is known that many of the characteristic features of plant development can be dramatically influenced by structurally simple phytohormones. It is evident now that there are five classes of plant hormones in higher plants, i.e. auxins, gibberellins, cytokinins, abscisic acid and ethylene. Each of them can elicit a remarkable variety of responses (Davis, 1987). Despite their demonstrable ability to modify cellular differentiation, the contribution of phytohormones to ontogeny and the mechanisms of their action remain to be determined (Davis, 1987). Approaches to this problem have prompted a longstanding debate concerning the relative importance of variations in phytohormone concentration versus differential sensitivity of different plant cells to particular phytohormones (Trewavas and Cleland, 1983; Weyers, 1984; Cannay, 1985). It has now become more widely accepted that the sensitivity of plant cells to hormonal signals is largely regulated by the status of the particular hormone receptors or binding proteins, and that the availability and status of both the hormones and their receptors are determinants of plant growth and development (Elliott *et al.*, 1988, 1990; Hall, 1986; Libbenga *et al.*, 1986; Stoddart, 1986; Napier and Venis, 1990).

1.1 Auxins

1.1.1 Biosynthesis

The sequence of events leading to the isolation and characterization of the major auxin

in higher plants, indole-3-acetic acid (IAA), can be traced back to the experiments carried out by Charles and Francis Darwin in 1880. These workers found that if the tip of a coleoptile was shaded with a small cap, then unilateral light would not induce a phototropic response. Since unilateral irradiation stimulated bending throughout the length of unshaded coleptiles, the Darwins concluded that the coleoptile tip perceived the light and transmitted some "influence" to the other tissues which induced differential growth (Darwin, 1881). Some years later, Boysen-Jensen (1910) demonstrated that the "influence" could pass through gelatin, and concluded that it was probably chemical in nature.

Attempts to extract the chemical into water proved unsuccessful; however, by modifying Boysen-Jensen's original procedure, Fritz Went (1928) succeeded in collecting diffusates from excised coleoptiles into agar blocks which, when placed asymmetrically on freshly decapitated coleoptiles, induced bending. This significant breakthrough not only confirmed the existence of a growth-promoting compound in coleoptile tips, but also resulted in the development of a quantitative bioassay for auxin. It was a short step from here to the identification of IAA as a compound with "auxin-like" activity (Kögl *et al.*, 1934) and later the demonstration that this was the primary auxin of higher plant tissues (Haagen-Smit *et al.*, 1946). Since then, there has been an intensive search for naturally occurring auxins. It is now known that auxins are present in all higher plants. IAA, however is not the only naturally occurring auxin, and both 4-chloro-indole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA) have been identified in this category (see Davis, 1987).

In the earlier years of auxin study, Bonner (1932) and Thimann (1935) found that the mould *Rhizopus suinus* could convert the amino acid tryptophan to IAA, raising the possibility that tryptophan was a primary precursor of IAA in higher plants. Later, the enzymes necessary for the conversion of tryptophan to IAA were found in spinach leaves and *Avena* coleoptiles (Skoog and Thimann, 1940; Wildman *et al.*, 1947). However, although a number of different pathways have been proposed to account for the biosynthesis of IAA from tryptophan, definitive evidence in support of any one of them has yet to be obtained. Fig. 1-1 illustrates the biosynthetic pathways by which tryptophan might be converted to IAA. Gordon and Nieva (1949) found that if leaf disks or crude extracts of pineapple leaves were incubated with tryptophan, tryptamine, or indolepyruvic acid, IAA was formed. They proposed that IAA could be formed from tryptophan via two different pathways. By either pathway, indoleacetaldehyde is formed and thus must be considered the immediate precursor of IAA in plants. One or both pathways have been detected in a variety of plant material (Lantican and Muir, 1967; Moore and Shaner, 1967; Phelps and Sequeira, 1967). Sherwin (1970) detected, in cucumber seedlings, the presence of tryptophan decarboxylase, an enzyme that enables these plants to convert tryptophan to tryptamine. Also, tryptophan transaminase activity has been detected in numerous plant species by Truelsen (1973). Indolepyruvic acid is thought to be derived from tryptophan by transamination. Indoleacetaldehyde is readily oxidized to form IAA.

Recently, there has been a great interest in the biosynthesis of IAA in *Agrobacterium tumefaciens*. *A. tumefaciens* is a bacterium which induces "crown gall" disease in many dicotyledonous species. Infection is associated with the development of neoplastic

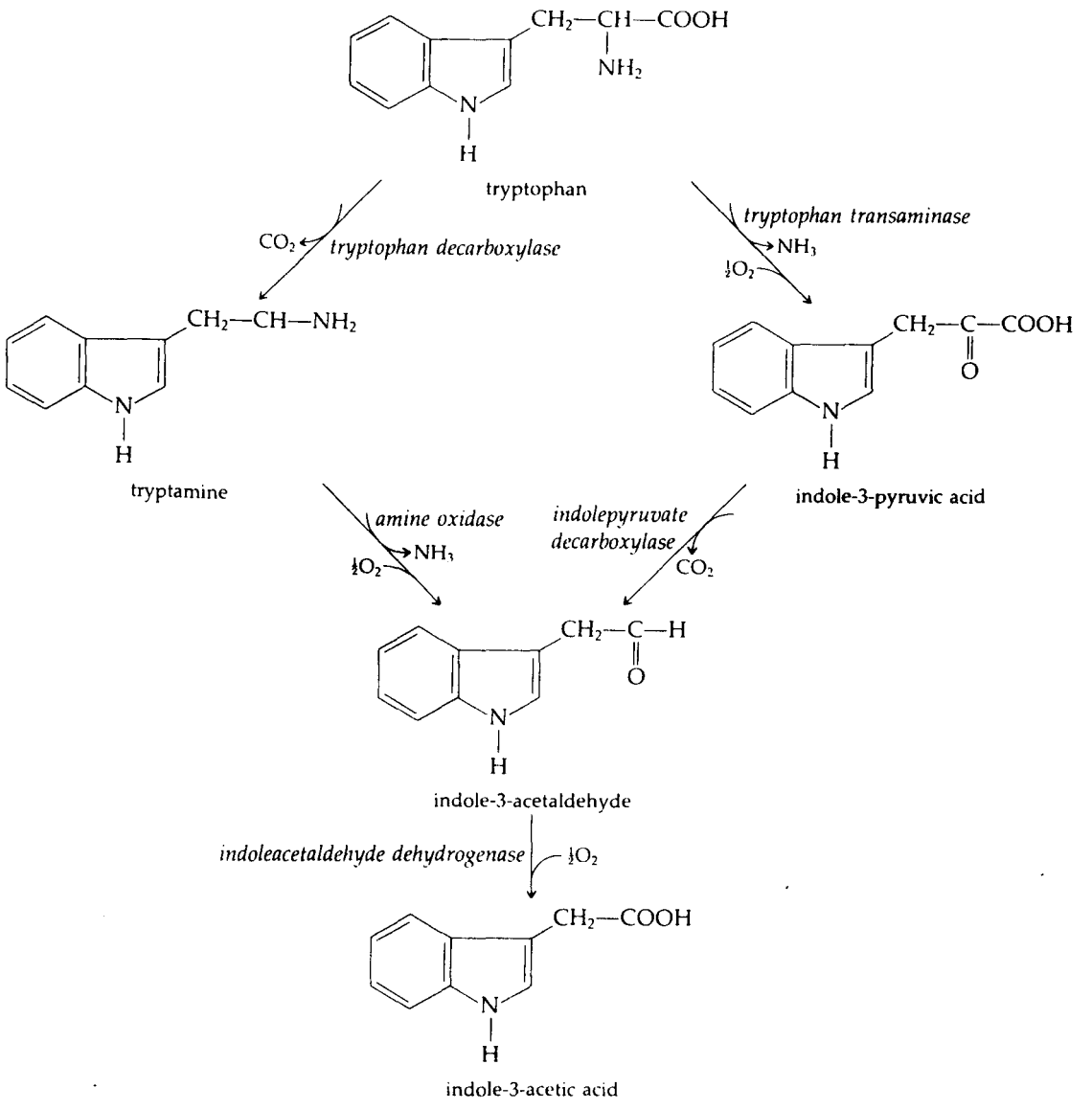


Fig. 1-1 Pathways of auxin synthesis from tryptophan

growth, and these tumours contain high concentrations of IAA. It has been demonstrated that, upon infection of *A. tumefaciens*, T-DNA (transforming DNA) of the Ti (tumour-inducing) plasmids become integrated into the genome of host plant. Two of the genes transferred encode enzymes that together produce IAA (Thomashow *et al.*, 1986). The first gene (gene 1) encodes tryptophan monooxygenase which has the ability to convert tryptophan to indoleacetamide. The second gene (gene 2) codes for indoleacetamide hydrolase, which converts indoleacetamide to IAA. The discovery of these genes has made it possible to engineer plants that produce high levels of IAA at tissue-specific sites. Clearly, such plants will make an important contribution to our understanding of the role of IAA in plant growth and development.

1.1.2 Transport

The concept of translocation of chemical messengers in higher plants was expressed in the nineteenth century (e.g. Sachs, 1880) and was confirmed by Went's discovery of auxin (Went, 1928) which was eventually shown to be indole-3-acetic acid (Haagen-Smit *et al.*, 1941). The polarity of auxin transport in cereal seedlings was established by the 1930s and later found to be a widespread feature of shoot and root tissues. Transport of IAA is saturable, occurs at a rate of approximately 10-15 mm hr^{-1} depending on the tissue, and is specifically inhibited by compounds such as 2,3,5-triiodobenzoic acid (TIBA) and N-1-naphthylphthalamic acid (NPA). These chemicals have therefore been routinely employed to probe the role of auxin transport in a particular developmental event. The balance of evidence from studies on root segments indicates that IAA transport in this tissue is predominantly acropetal.

Therefore a flux of auxin is maintained throughout the plant from shoot to root apex.

Polar transport of IAA in both shoot and root tissues is maintained against a concentration gradient and is inhibited by anaerobic conditions. These properties of the system indicate that polar transport has a requirement for metabolic energy; this energy might be consumed by events related to either the uptake or efflux processes. Because of the difficulties of examining these components in a multicellular tissue, progress in elucidating the mechanism of IAA transport has come primarily from work on populations of single cells. Investigations carried out by Rubery and Sheldrake (1974) on crown gall cell suspension cultures of *Parthenocissus*, and by Raven (1975) on the giant alga *Hydrodictyon*, led to the formulation of the "chemiosmotic polar diffusion hypothesis" of IAA transport (Goldsmith, 1977). This simple theory is centred on two basic properties of the IAA molecule. Firstly, undissociated molecules of the auxin are hydrophobic and therefore readily traverse lipid membranes. Secondly, since IAA is a weak acid, its rate of dissociation to form the lipophobic anion IAA^- increases with rising pH. From a consideration of these facts, it can be predicted that plant cells surrounded by an acidic environment, such as the cell wall, would readily take up IAA. However, once inside the cell, the auxin would dissociate in the more basic environment of the cytoplasm and the IAA^- anions would become effectively trapped. If an IAA^- carrier was localized within the plasma membrane, this would reduce anion accumulation by catalysing its efflux down a concentration gradient. Polarity of auxin transport would be achieved by the maintenance of an asymmetric distribution of efflux sites within the cell, and ATP would be required to sustain the pH and electrical gradients necessary to drive the transport process (Fig. 1-2). Since this hypothesis was

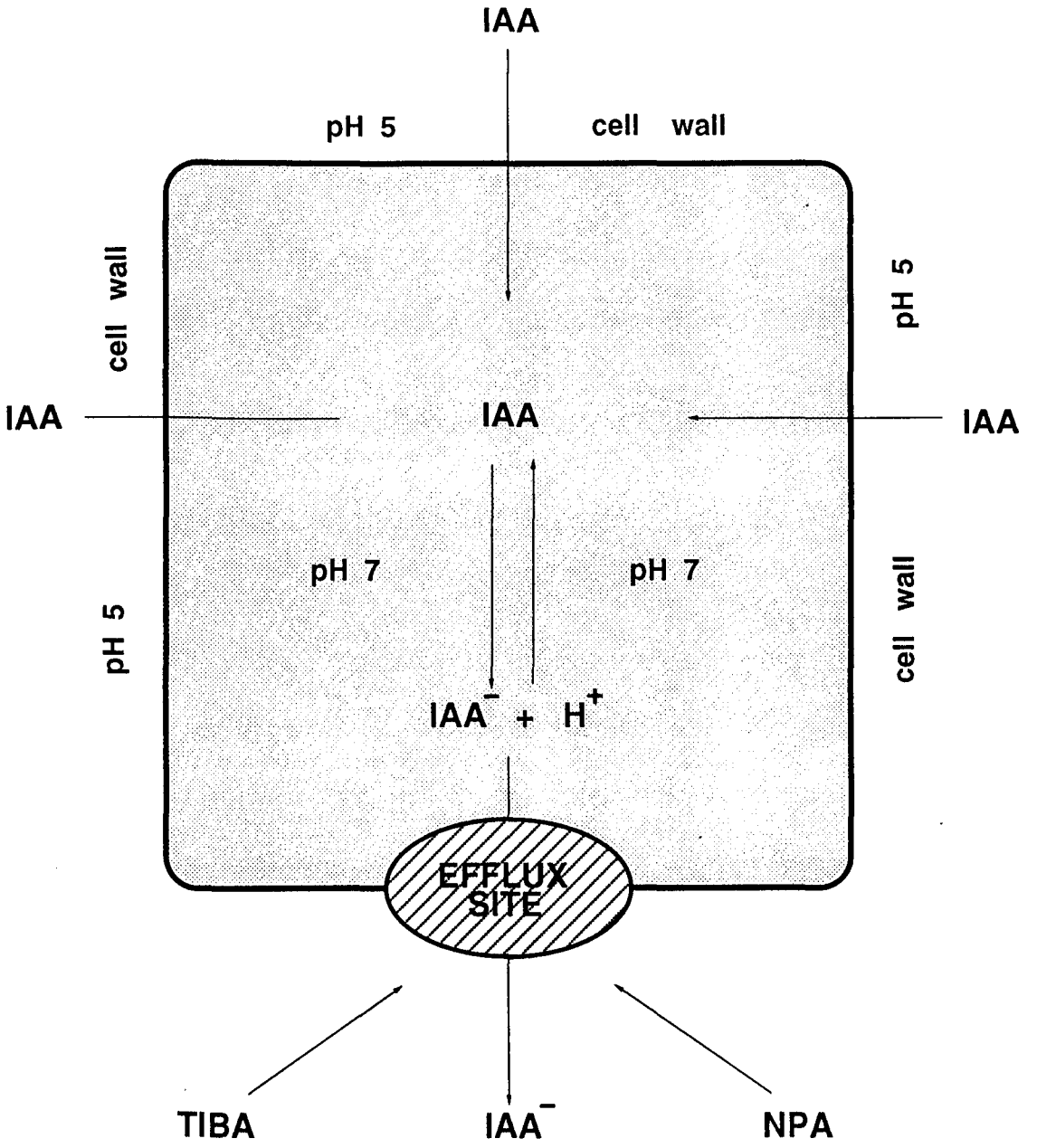


Fig. 1-2 The chemiosmotic hypothesis of IAA transport

proposed, auxin efflux carriers have been detected in a variety of tissues which exhibit polar transport. Furthermore, kinetic studies of IAA movement in *Cucurbita pepo* hypocotyl segments indicate that the efflux carrier is specifically blocked by TIBA and NPA, although these chemicals appear to bind to a different site on the carrier than IAA (Benning, 1986; Hertel, 1983, 1986; Sabater and Rubery, 1987). The NPA binding protein has been purified and monoclonal antibodies used to identify its cellular location in pea stems by immunofluorescence techniques (Jacobs and Gilbert, 1983). In accord with the predictions made by the chemiosmotic hypothesis, NPA-binding proteins are specifically associated with the basal end of cells, and these are situated adjacent to the vascular tissue (Jacobs and Short, 1986). NPA is not a natural constituent of plant cells, and therefore a question that has been frequently posed is the nature of the molecule which binds to the NPA receptor *in vivo*. Jacobs and Rubery (1988) have shown that certain flavanoids such as quercertin can displace bound NPA from plant tissues and inhibit polar auxin transport. Therefore it is plausible that certain phenolics could play a role in regulating IAA transport in plant tissues. It seems entirely possible that other, high affinity endogenous phytotropins with somewhat different properties may be found, by analogy with, for example, the different types of natural ligand found for benzodiazepine receptors (Sangameswaran *et al.*, 1986).

1.2 Binding studies

Specific attention has been paid in the last 15 years to research on hormone-binding proteins of plants. The general area of plant growth regulator receptors has been thoroughly reviewed by Venis (1985), Hall (1986), Libbenga *et al.* (1986), Stoddart

(1986) and Napier and Venis (1990).

1.2.1 Receptor criteria

A receptor is a specific cellular recognition site (e.g. for a hormone, drug or neurotransmitter) that binds the ligand and in consequence instructs the cell to respond in the appropriate manner to the particular chemical signal. The high specificity of the recognition process can probably only be accommodated in a macromolecular structure and all known hormone receptors are proteins. When studying a putative receptor system, it is necessary to distinguish receptors and hormone-binding nonreceptor proteins, i.e. metabolic enzymes, transport and storage proteins, etc. This can be done with the aid of basic criteria of receptors:

(1) Binding should be reversible, of high affinity, and of finite capacity, in order that the physiological effect can be regulated and be responsive to changes in hormone concentration.

(2) The saturation range of binding should be consistent with the concentration range over which the physiological response saturates.

(3) Binding specificity for different hormone analogues should be approximately in accordance with the relative biological activities of the compounds. A given receptor should not bind hormones of another class.

(4) Binding may be confined to hormone-responsive tissues.

(5) Binding should lead to a hormone-specific biological response.

On the basis of these criteria, binding experiments are performed to determine the presence or absence of a putative receptor in an extract from a tissue. Criteria (1)-(3) are usually investigated. Criterion (4) is less often addressed, and (5), although of greatest significance, is the most difficult to establish and therefore rarely studied.

1.2.2 Ligand-receptor interactions

Biological responses to a ligand are generally graded, that is, there is a relationship between the concentration of a ligand and the magnitude of the response. If each receptor binds a single ligand molecule according to the equilibrium law, the interaction can be described by:

$$L + R \underset{k_{-1}}{\overset{k_{+1}}{===}} LR \qquad (1)$$

where L is ligand, R the unoccupied receptor sites, LR the ligand-receptor complex, k_{+1} the association rate constant, and k_{-1} the dissociation rate constant.

At equilibrium:

$$[L][R]k_{+1} = [LR]k_{-1} \qquad (2)$$

$$\frac{[L][R]}{[LR]} = \frac{k_{-1}}{k_{+1}} = \frac{1}{Ka} = Kd \qquad (3)$$

where Kd and Ka are, respectively, the equilibrium dissociation and association constants. Kd is equivalent to the concentration of ligand at which half of the binding sites are occupied.

When equations for ligand-receptor interactions are derived, the following assumptions are made:

(1) the ligand is in homogeneous form;

(2) the ligand is univalent (that is, one ligand molecule reacts with only one binding site);

(3) full equilibrium is reached, and both bound and free ligand can be separated for quantitation without affecting this equilibrium;

(4) the observed response is directly proportional to the number of receptors occupied;

(5) no interactions exist between binding sites;

(6) ligand-receptor interactions occur as simple, reversible reactions.

Returning to equation (3), we introduce the term [Rt] to denote the total concentration of receptor sites in the tissue:

$$[Rt] = [R] + [LR] \qquad (4)$$

Therefore,

$$[R] = [Rt] - [LR] \qquad (5)$$

Substituting for [R] from equation 5 in equation 3,

$$Kd = \frac{[L]([Rt]-[LR])}{[LR]} \qquad (6)$$

therefore

$$Kd = \frac{[L][Rt]}{[LR]} - \frac{[L][LR]}{[LR]} \qquad (7)$$

Cancelling the [LR]s in the second term,

$$Kd = \frac{[L][Rt]}{[LR]} - [L] \qquad (8)$$

rearranging,

$$Kd + [L] = \frac{[L][Rt]}{[LR]} \qquad (9)$$

Therefore

$$[LR] = \frac{[L][Rt]}{Kd + [L]} \qquad (10)$$

Converting to standard binding terminology, [LR] = B = concentration of bound ligand; [Rt] = n = total binding site concentration; [L] = F = concentration of unbound (free) ligand, then:

$$B = \frac{nF}{Kd + F}$$

Commonly used linear transformations for graphic representation and derivation of binding constants are:

$$\frac{B}{F} = \frac{n}{Kd} - \frac{B}{Kd}$$ Scatchard (1949) plot (B/F vs B)

$$\frac{1}{B} = \frac{Kd}{nF} + \frac{1}{n}$$ Double reciprocal plot (1/B vs 1/F)

The binding constants Kd and n can be obtained from the slope or intercept of these plots, respectively (Fig. 1-3). The Scatchard plot is very useful as it does not depend on measuring a response but simply on the amount of bound and free ligand, and gives two very useful parameters of the receptor. These are Kd, the dissociation constant, which is a measure of binding affinity; and n, the number of binding sites associated with the tissue. Other graphical methods and discussion of more complex receptor models can be found in Boeynaems and Dumont (1980).

In order to estimate Kd and n, the values of B (the concentration of bound ligand) and F (the concentration of free ligand) must be measured. These values must be determined over a range of total ligand concentrations which fall below those giving receptor saturation. This is most readily achieved by the use of radiolabelled PGR with

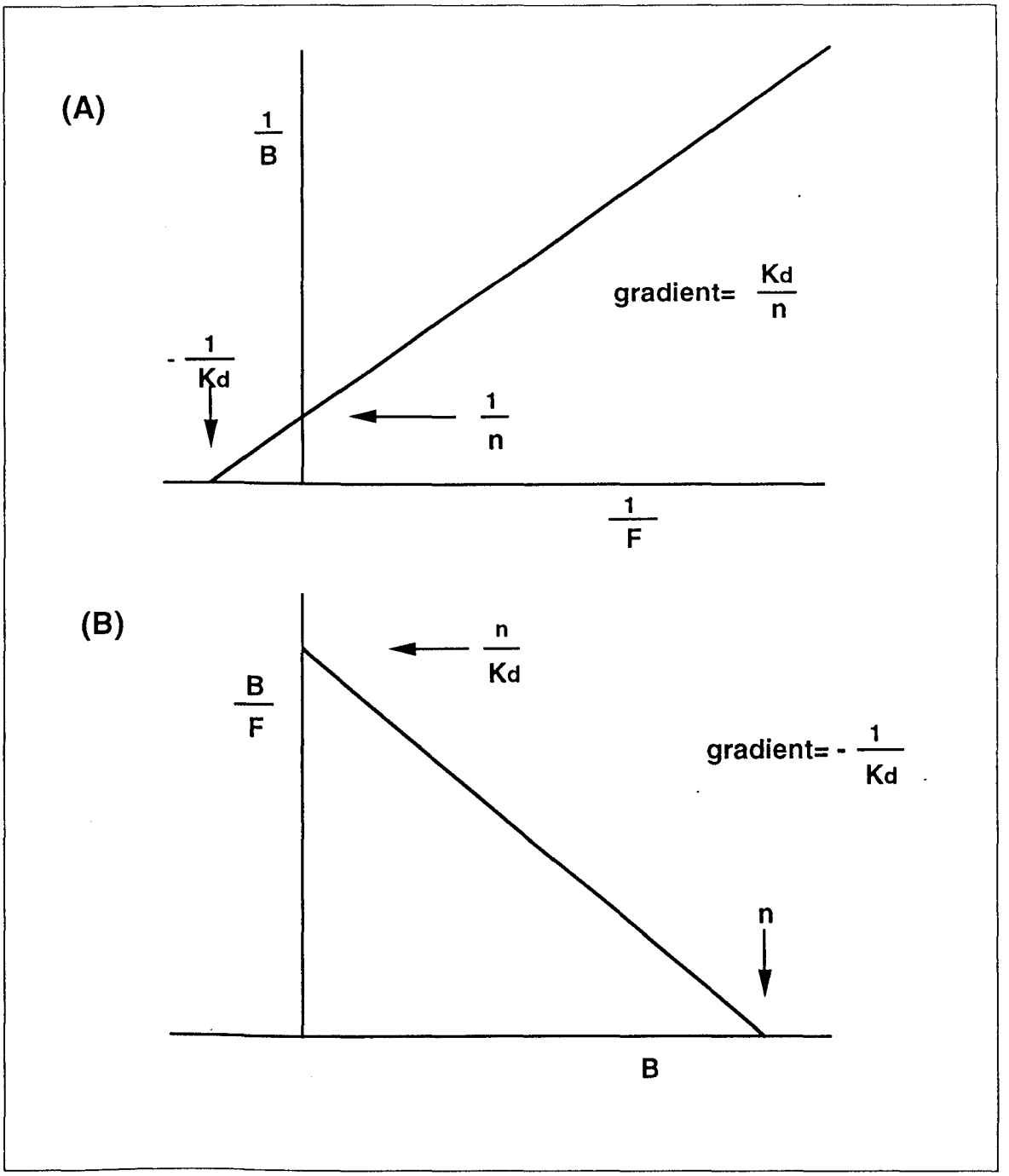


Fig. 1-3 Linear transformations of binding data
(a) Double reciprocal plot
(b) Scatchard plot

high specific activity. The preferred concentration range is one that will saturate from about 10 to 90% of the receptor, corresponding to values from about 0.1 - 10 times the Kd or receptor concentration.

Either of two general strategies can be used. The first is to increase the concentration of radioactive ligand at constant specific activity (Fig. 1-4a). The second general method, more commonly used because it conserves expensive or scarce radiolabel, is to use a single, low concentration of radioactive hormone and add increasing amounts of unlabelled hormone (Fig. 1-4b). Both soluble and membrane-bound proteins can be assayed for binding activity in this way, although the experimental protocols vary.

1.2.3 Detection and quantitation of binding sites in particulate preparations

Bound and free ligand are generally separated by centrifugation or filtration methods.

(a) Centrifugation: This is normally the most convenient and accurate method. It can be used to provide a rapid and efficient way to separate the pellet containing bound ligand from the supernatant medium. However, contamination by free ligand from the supernatant may produce errors.

(b) Filtration: Special filters composed of inert cellulose esters or other polymers, which can be obtained in a range of pore sizes, provide a rapid method of separating cells or membranes containing bound ligand from incubation medium containing the unbound or free ligand (Fig. 1-5). This technique is simple but problems can occur

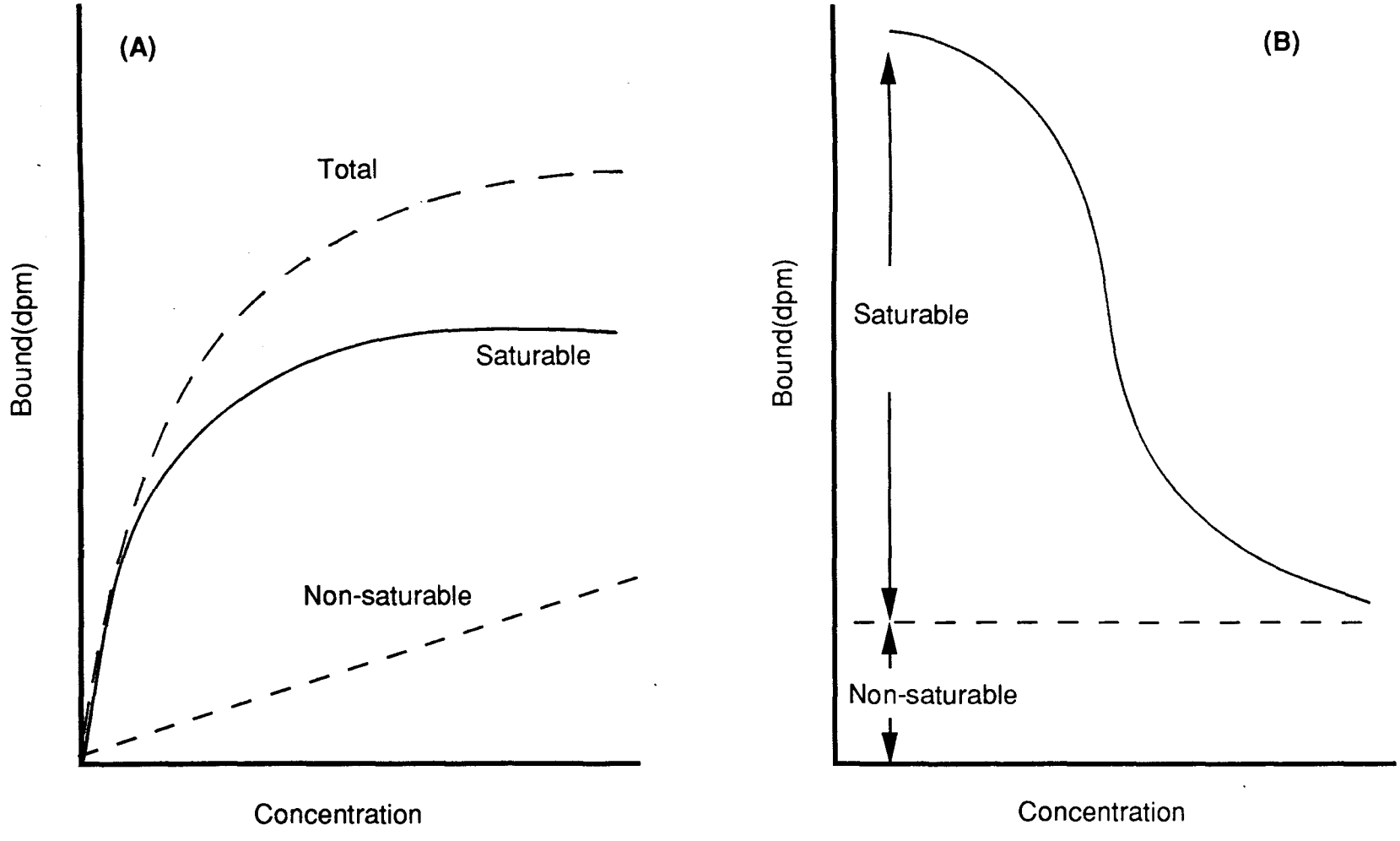


Fig. 1-4 Two general strategies used for binding assay

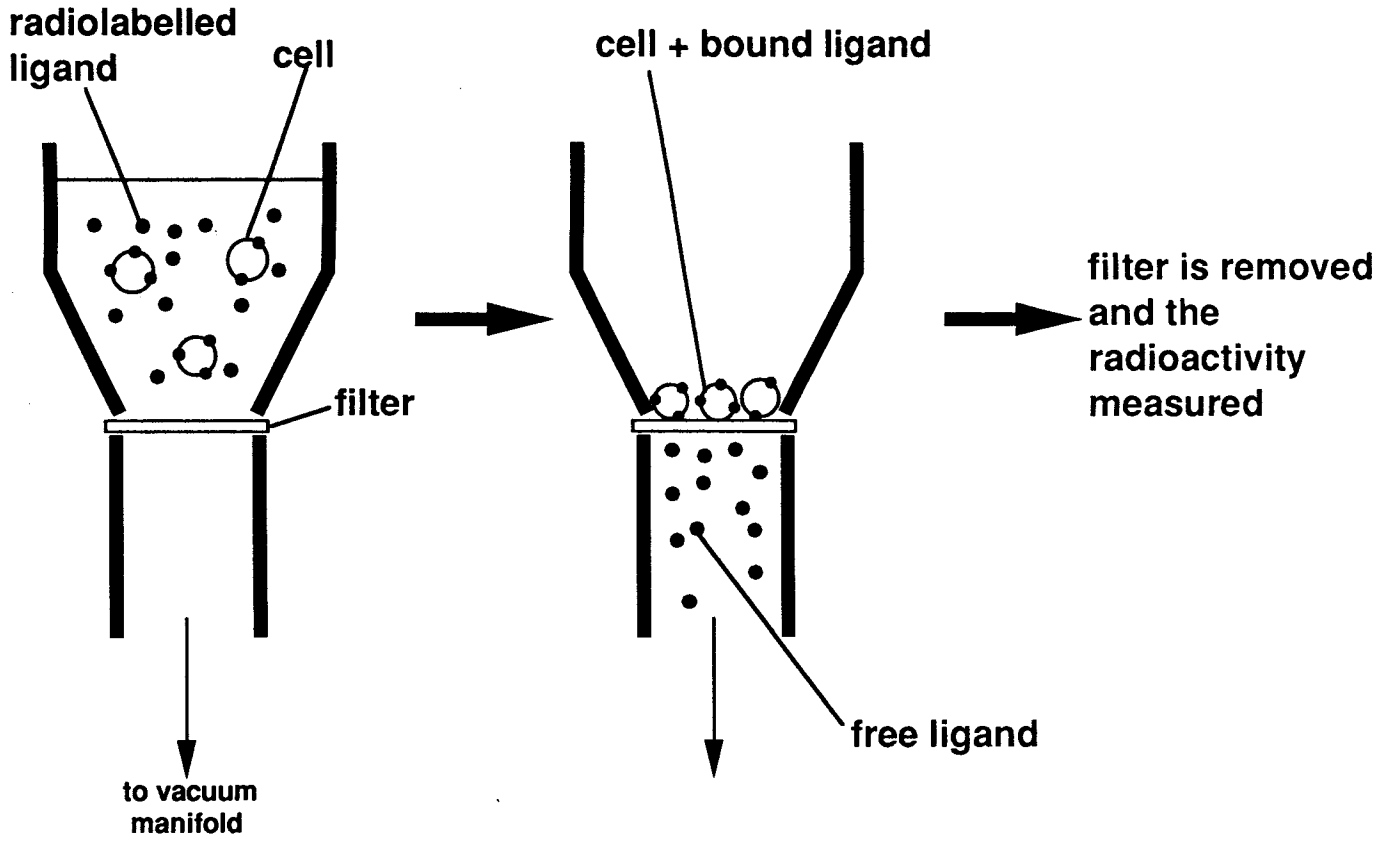


Fig. 1-5 Filtration method for separating ligand bound to cells or membranes from free ligand

when the filter used has a high affinity for free ligand.

(c) Other methods: Additional methods that can and have been used to measure binding to particulate preparations are equilibrium dialysis and gel filtration. However, these techniques are more commonly used with soluble and solubilized receptors.

1.3 Binding proteins for auxin transport inhibitors

Auxins are transported by specific carriers (see section 1.1.2), one of which has been characterized by its ability to bind the synthetic auxin transport inhibitor N-1-naphthylphthalamic acid (NPA). It is generally believed that NPA binds to an auxin-specific, saturable efflux carrier. This carrier is assumed to bind auxin at the cytoplasmic site of the plasma membrane, transport it through the membrane and release it into the cell-wall free side (Morris, 1988; Libbenga and Mennes, 1987).

The NPA binding protein was first detected in maize membrane fractions (Lembi *et al.*, 1971). Binding was found to be correlated with plasma membrane content of the fractions, it was reversible and of high affinity, with a Kd of approximately 2 x 10^{-8} mol dm^{-3}. There was a good correlation between binding affinity and the effect of NPA on auxin transport (Thomson *et al.*, 1973), though the values of affinities vary somewhat (Lembi *et al.*, 1971; Thomson, 1972; Normand *et al.*, 1975; Trillmich and Michalke, 1979; Sussman and Gardner, 1980; Katekar *et al.*, 1981). A similar conclusion was reached when membranes from tobacco cell suspension cultures were used. The mean Kd value from two independent methods was 3 x 10^{-9} mol dm^{-3}, an

affinity about ten times greater than that observed in maize (Maan *et al.*, 1985). Furthermore, NPA derivatives were found to displace ^{3}H-NPA from the binding sites in a manner consistent with their activities as transport inhibitors (Thomson and Leopold, 1974; Katekar and Geissler, 1975, 1977; Katekar, 1976, 1987; Katekar *et al.*, 1981). However, bound ^{3}H-NPA was not displaced by physiologically active concentrations of another inhibitor of polar auxin transport, 2,3,5-triiodobenzoic acid (TIBA). It was found that about 50% displacement of bound ^{3}H-NPA by TIBA occurred at 10^{-5} mol dm^{-3} (Depta *et al.*, 1983; Katekar, 1987) or 10^{-4} mol dm^{-3} (Sussman and Goldsmith, 1981). Nevertheless this order of binding affinity has been considered to be too low to account for activity of TIBA on polar transport: the I_{50} of inhibitory effect of TIBA on auxin transport is about 2-3 x 10^{-6} mol dm^{-3} (Thomson *et al.*, 1973; Depta *et al.*, 1983). Therefore, it has been suggest that NPA and TIBA inhibit transport at distinct sites.

IAA itself is not particularly effective at displacing ^{3}H-NPA from its binding site in membrane preparations. Even at 5 x 10^{-4} mol dm^{-3}, no interaction with IAA could be observed in maize (Sussman and Gardner, 1980). However, the auxin did displace NPA in binding protein solubilized from the membrane preparation with detergent (Sussman and Gardner, 1980). A binding protein for NPA was solubilized from maize coleoptile membranes using the non-ionic detergent Triton X-100, yielding preparations with NPA binding kinetics very similar to those seen in the native state. The most significant change in properties upon solubilization was the ability of IAA (10^{-6}-10^{-5} mol dm^{-3}) to displace ^{3}H-NPA from the binding sites; the affinity for NAA was also enhanced, by at least an order of magnitude (Sussman and Gardner, 1980). This was confirmed by

Jacobs and Gilbert (1983) who has demonstrated that both saturable NPA binding and the IAA-sensitive component were inhibited by antibodies from an active clone (Table 1.1). This indicated that the NPA binding site recognized by the antibody can also interact with IAA and therefore supports the immunofluorescence evidence that auxin efflux carriers are preferentially located at the base of transporting cells, as required by the chemiosmotic hypothesis. This was the first localization of a receptor molecule in plant cells and was the first report using monoclonal antibodies to label plant tissue.

Table 1.1 Comparison of the effects of active and inactive antibodies on specific and IAA-sensitive NPA binding to solubilized pea membranes (from Jacobs and Gilbert, 1983)

Treatment	^{3}H-NPA bound	Radioactivity (cpm)	
		Specific NPA binding (A-B)	IAA-sensitive NPA binding (A-C)
Inactive supernatant			
A. 10^{-9} mol dm^{-3} ^{3}H-NPA	1704		
B. 10^{-9} mol dm^{-3} ^{3}H-NPA + 10^{-5} mol dm^{-3} NPA	952	752	
C. 10^{-9} mol dm^{-3} ^{3}H-NPA + 10^{-5} mol dm^{-3} IAA	1178		526
Active supernatant			
A. 10^{-9} mol dm^{-3} ^{3}H-NPA	1494		
B. 10^{-9} mol dm^{-3} ^{3}H-NPA + 10^{-5} mol dm^{-3} NPA	1141	353	
C. 10^{-9} mol dm^{-3} ^{3}H-NPA + 10^{-5} mol dm^{-3} IAA	1614		-120

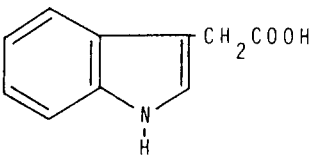
1.4 Auxin binding proteins

Auxin-binding protein is the best-known receptor system in plants (Libbenga and Mennes, 1987). In addition to the naturally occurring auxin, many synthetic auxin analogues (Fig. 1-6) have been synthesized and structure-activity rules have been formulated (Kaethner, 1977; Farrimond *et al.*, 1978; Katekar, 1979). A substance is called an anti-auxin if it reversibly inhibits growth induced by an auxin (Housley, 1961). To study exclusively auxin-binding proteins, the experimental systems most widely used are those in which auxin seems to be a major limiting factor.

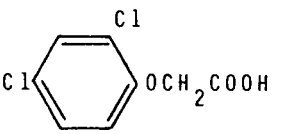
The most intensive research has been devoted to study of the auxin-binding proteins of corn coleoptiles (Hertel *et al.*, 1972; Batt and Venis, 1976; Venis, 1977; Ray *et al.*, 1977a,b; Dohrmann *et al.*, 1978; Murphy, 1980; Löble and Klämbt, 1985a,b; Venis, 1987; Inohara *et al.*,1989; Hesse *et al.*, 1989; Tillmann *et al.*, 1989; Napier and Venis, 1990; Yu and Lazarus, 1991). Several classes of auxin-binding proteins have been identified and partially characterized. These comprise membrane-bound auxin-binding proteins and cytoplasmic/nuclear soluble auxin-binding proteins. These classes will be briefly reviewed.

1.4.1 Membrane-bound auxin binding proteins

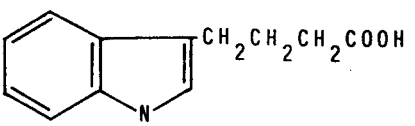
Auxin binding proteins in maize coleoptile membranes were first studied by Hertel *et al.*, (1972). They successfully introduced the centrifugation method for separation of bound and free ligand, experimental determinations of non-specific binding and



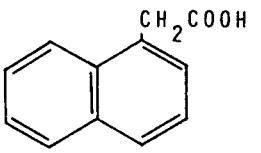
(1) IAA MW 175.19



(2) 2,4-D MW 221.04



(3) IBA MW 203.24



(4) NAA MW 186.21

Fig. 1-6 Structures of the naturally occurring auxin IAA and three synthetic auxins, IBA, NAA, and 2,4-D

Scatchard analysis. Membrane-bound auxin-binding proteins have been found in cells of many tissues. Based on binding specificities and buoyant densities in sucrose gradients, three auxin-binding proteins, designated I, II and III, have been described.

Class I binding protein (protein I) has been assigned to the endoplasmic reticulum (Normand *et al.*, 1975), whilst protein II has been suggested to be located at the tonoplast. Protein I and protein II could be distinguished kinetically, either by assaying in the presence (protein II) or absence (protein I) of PAA or, as described by Batt and Venis (1976), by assaying membrane fractions resolved on sucrose gradients. Protein I and II were characterised in maize coleoptile homogenates (Ray *et al.*, 1977; Dohrmann *et al.*, 1978). Using the same material, Dohrmann *et al.* (1978) obtained indications of a third auxin-binding protein, protein III. Protein III appears at 30 - 38% sucrose, coinciding with a plasma membrane marker (NPA-binding). In zucchini membrane preparation, protein III binding has been characterized, with a Kd of 1-2 x 10^{-6} mol dm^{-3} for IAA. It has been suggested that protein III is a plasma membrane-bound auxin transport protein (Jacobs and Hertel, 1978). Based on experimental observations, it is now known that over 90% of auxin-binding proteins is located on ER (Ray, 1977; Venis, 1985).

The three classes of binding proteins show a difference in affinity for various analogues. The affinity of site I for NAA, IAA and PAA is substantially higher than the affinity of site II (Batt and Venis, 1976; Ray *et al.*, 1977b; Dohrmann *et al.*, 1978; Murphy, 1980). Both protein I and protein II have a relatively low affinity for 2,4-D, whereas protein III has a relatively high affinity for 2,4-D and a relatively low affinity

for NAA and IAA (Dohrmann *et al.*, 1978; Jacobs and Hertel, 1978). However, for all three proteins, the binding is most rapid at 0°C and the optimal pH of binding is below 7 (pH = 4-6.5).

The auxin-binding protein from maize coleoptiles has been solubilized from their membranes by an acetone method and purified by FPLC and affinity chromatography (Venis, 1977, 1980; Löbler and Klämbt, 1984). Auxin binding activity elutes from gel filtration columns at around 40-45 kD and has an isoelectric point in the range pI 4.5-5.2 (Cross and Briggs, 1978; Venis, 1980; Murphy, 1980; Tappeser *et al.*, 1981). Monospecific antibodies have been raised which recognize the affinity purified auxin binding protein. SDS gel electrophoresis of the immunoprecipitated auxin binding protein has revealed that the polypeptide has a molecular weight of 20-22 kD (Löbler and Klämbt, 1985a, b). These data suggest that the native protein is either a dimer, or a monomer which aggregates during gel filtration. It is believed that this protein represents ABP-I (Löbler and Klämbt, 1985a, b; Napier *et al.*, 1988; Napier and Venis, 1990).

Taking a slightly different approach, Napier *et al.* (1988) reported purification work on the same material. It involed three chromatographic steps, i.e. anion exchange chromatography (DEAE Bio-Gel A), gel filtration (Sephacryl S-200) and high-resolution anion exchange chromatography (Mono Q). Monitoring of the Mono Q elution profile by SDS-PAGE revealed a predominant 22-KD polypeptide at the binding peak. A minor band at 21-KD eluted slightly earlier and was consistently associated with the major 22-KD species.

Easy solubilization and reliable protocols for purification of ABP-I have greatly facilitated the study of ABP using immunobiological techniques. Gel slices from native PAGE containing pure ABP were used to generate a polyclonal antiserum. When this antiserum was used to probe nitrocellulose blots of a post-DEAE ABP fraction after SDS-PAGE, a band at 22-KD was selected. The 21-KD minor band was also recognised by the serum. Post-Mono Q fractions were used to immunise rats to raise monoclonal antibodies (mAbs). Two of the resulting five mAbs specifically recognised the major 22-KD-binding protein polypeptide whilst the other three recognized, in addition, a minor 21-KD species (Napier *et al.*, 1988).

Rapid ABP separation by SDS-PAGE in conditions designed to inhibit proteolysis yielded only the 22-KD polypeptide, but the two polypeptides showed strong similarities in terms of chromatographic properties, glycosylation and immunoreactivity. It is likely, therefore, that the 21-KD species is a breakdown product of the 22-KD species. Studies on the digestion of polypeptides with endoglycosidase H indicated that all the mAbs recognized the polypeptide rather than the glycan side chain and the polyclonal antiserum also recognises deglycosylated binding protein (Napier *et al.*, 1988).

Determination of the amino acid sequence of the purified maize protein allowed the synthesis of two oligonucleotides (Inohara *et al.*, 1989). Preliminary screening of a cDNA library from maize shoots resulted in the isolation of a partial length cDNA that was then used to reprobe the same library. From the clones isolated, including one of full length, the complete nucleotide sequence encoding the auxin-binding protein was obtained and the primary amino acid sequence deduced. The sequence has been

confirmed by others (Hesse *et al.*, 1989; Tillmann *et al.*, 1989; Yu and Lazarus, 1991).

The ABP has a signal peptide of 38 residues which, although very long, does conform to the general rules described by von Heijne (von Heijne, 1985) for signal peptide composition. This peptide is cleaved from the whole translation product between two serines to give a mature protein of 163 residues. The signal peptide contains the only stretch of hydrophobic amino acids within the sequence that is long enough to span a lipid bilayer as an α-helix. That the protein has always been extracted with the membranes is due, at least in part, to the C-terminal tetrapeptide, KDEL, which is known to be responsible for the retention of proteins within the lumen of ER (Munro and Pelham, 1987). There is a single *N*-glycosylation site within the sequence, consistent with results obtained by digestion with endoglycosidase H (Napier *et al.*, 1988). The glycan is of the high-mannose type that is characteristically added to proteins within the ER (Hesse *et al.*, 1989).

Northern hybridization analysis revealed a single mRNA species of approximately the same size as the cDNA isolated. Further studies from the same research group by using a highly purified $IgG_{anti\ ABP}$, an identical ABP cDNA clone was selected from a λ*gt* 11 cDNA library from corn coleoptiles (Tillmann *et al.*, 1989). The cDNA is the same as that selected by oligonucleotides designed from the amino acid sequence of purified main component of ABP (Hesse *et al.*, 1989). Molecular cloning and structural analysis of the genes coding for ABP will greatly help our understanding of its roles in the action of auxin.

1.4.2 Cytoplasmic/nuclear auxin binding proteins

One class of specific high-affinity hormone-binding proteins which is present in both the cytoplasm and the nucleus is often regarded as essential for a cell to respond to a hormonal signal, especially when the response includes alterations in expression of the genome.

The most completely characterized cytoplasm/nuclear soluble auxin binding protein (sABP) has been isolated from tobacco callus cultures. High affinity binding (Kd 10^{-8} mol dm^{-3}) was detected using high specific activity tritiated IAA and a dextran-charcoal assay (Oostrom *et al.*, 1975). The concentration of binding sites was consistently very low (ca. 0.01 pmol mg^{-1} protein). In common with the non-membrane-bound proteins in tobacco, optimum binding was observed at 24-30°C, after 25-30 min incubation, but with a higher pH optimum (pH 7.5-7.8) (Oostrom *et al.*, 1980). The binding proteins were partially purified by gel permeation and by ion exchange chromatography. Van der Linde *et al.* (1984) succeeded in enhancing the purification of this binding protein. When boric acid buffers at pH 6.8 were used for homogenization and gel filtration, contamination of the cytosol by polyphenols was substantially reduced. The active protein fraction obtained by this method eluted from gel filtration columns in the range 150-200 KD, somewhat lower than the previous apparent value. However, the progress of this research was hampered by the rather poor reproducibility of the detection of this IAA-binding protein and by its very low concentration in cultured tissues. Van der Linde *et al.* (1984) found that the amount of high-affinity IAA binding in crude cytosol preparations was increased if Mg^{2+} and ATP and/or a phosphatase substrate

were included in the binding assay. These results suggest that an important source of preparative variability may be receptor dephosphorylation.

RNA synthesis by isolated tobacco nuclei was stimulated by addition of soluble auxin-binding protein in an IAA-dependent manner (Van de Linde *et al.*, 1984). Although it was not clear whether this was an overall stimulation or it was a stimulation of the transcription of specific sets of genes, the results had been regarded as a first step to demonstrate an involvement of this protein in the auxin-mediated regulation of transcription (Libbenga *et al.*, 1986). Comparable results were obtained with a 2,4-D dependent cell suspension line from *Nicotiana tobacum* (Bailey *et al.*, 1985). Moreover, it was found that in early-stationary phase cells most specific auxin binding activity was present in the cytosol, whereas in rapidly dividing log-phase cells most specific auxin-binding was present in salt extracts of isolated nuclei. This observation is extremely interesting in that it resembles the apparent translocation of occupied steroid receptors to the nuclei in hormone-activated target tissues. Two classes of soluble auxin-binding protein have been reported in cultured soybean cells (Herber *et al.*, 1988; Jacobsen *et al.*, 1987) and mung bean seedlings (Sakai *et al.*, 1986). Later, Sakai's group noted that, although addition of sABP-I or sABP-II alone to the isolated nuclei stimulated RNA synthesis (Sakai *et al.*, 1986), the translatable transcripts in poly(A)$^+$ RNA from these nuclei did not show marked differences on 2-dimensional electrophoresis (Kikuchi *et al.*, 1989). It appeared that sABP-I or sABP-II alone stimulated the synthesis of the same species of mRNA that were synthesized in the control nuclei. However, the addition of IAA together with sABP-I or sABP-II to the nuclei resulted in the appearance of novel translatable transcripts that were not detected

otherwise. The levels of two peptides which had the same molecular weights and isoelectric point as these specifically translated peptides also increased in the translation products of poly(A)$^+$ RNA from IAA-treated or 2,4-D-treated sections of mung bean hypocotyls (Kikuchi *et al.*, 1989). These results suggested that the mRNAs synthesized in the isolated nuclei in the presence of sABP plus IAA were also synthesized in tissues treated with auxin. Auxin was also shown to induce changes in specific translatable mRNA species in tobacco (Van der Zaal *et al.*, 1987) for which there are now cDNA probes (Mennes *et al.*, 1990) and so it should be possible to test whether the soluble auxin-binding protein can alter the level of these particular mRNAs. Heterologous probes have also been developed for mRNAs that are rapidly upregulated by auxin (e.g. McClure *et al.*, 1989).

It is interesting to note in the work on mung bean that , in the transcription system composed of isolated nuclei, auxin interacts with soluble ABPs and stimulates the expression of specific genes which are also activated in tissues treated with auxin. The interaction of IAA with two different sABPs activated two different genes. One of the mechanisms of action of IAA may involve a direct interaction between a soluble receptor protein, such that the resultant auxin-receptor complex can subsequently recognise the promoter region of specific gene(s). Binding of sABP-I to DNA is currently being studied by Sakai's group (Sakai, 1992).

1.5 Plant cell suspension cultures

Suspension cultures are usually initiated by transferring established callus tissues to a

liquid medium. They have also been started from sterile seedlings of imbibed embryos by breaking up the soft tissues in a hand-operated glass homogenizer and then transferring the homogenate, containing intact living cells, dead cells and cell debris, to moving liquid medium. However, no suspension culture has yet been shown to be composed entirely of free-floating single cells. Even the most dispersed cultures so far established, such as those of sycamore, consist of cell aggregates as well as single cells.

In general, media suitable for growing callus cultures for a particular species are also suitable for growing suspension cultures, providing that agar is omitted. Nevertheless, in some cases suspensions are more exacting in their requirements; for example, the concentrations of auxins and cytokinins are often more critical.

Batch culture is one of the systems currently being used for cell suspension cultures. Such a culture is a closed system where the cell material grows in a fixed volume of medium which is agitated to maintain the even distribution of free cells and cell aggregates and to promote adequate gas exchange with the air. This agitation may be achieved by slowly rotating the cultures (Steward *et al.*, 1952), by shaking the cultures on orbital platform shakers (Rajesekhar *et al.*, 1971), by spinning (Lamport, 1964; Short *et al.*, 1969) or by stirring the cultures (Melchers and Engelmann, 1955; Wilson *et al.*, 1971). During incubation the amount of cell material increases for a limited period of time and reaches a point of maximum yield. If the culture is to be maintained, a small volume of the suspension must be removed and used to inoculate a flask of fresh medium. The growth pattern will then be repeated to yield a similar

amount of material. In this way the culture can be continuously maintained and propagated by successive inoculations at appropriate intervals into fresh medium.

Since Muir (1953) demonstrated that cells of tobacco and *Tagetes erecta* can be cultured in the form of cell suspensions, similar suspensions were obtained from carrot root explants and callus tissues of *Picea glauca*, *Antirrhinum majus* and many other species. Subsequenly it was shown by Nickell (1956) that the techniques primarily designed for the culture of micro-organisms could be used for growing relatively large quantities of cell suspensions. Plant cell suspension cultures have been used to study the biochemical basis of growth and differentiation in plants, they can also be used to identify the specific factors which regulate the onset and termination of cell division and cell expansion, as in such a system many of the complex cellular interactions occurring in plant tissues are essentially eliminated (Elliott *et al.*, 1982).

The growth cycle of batch cultured cells can be described by an S-shaped curve which consists of five distinct growth phases (Fig. 1-7):

1. Lag phase (cell numbers are constant);

2. Exponential phase (specific growth rate increase);

3. Linear phase (specific growth rate is constant);

4. Phase of progressive decelaration (specific growth rate declines);

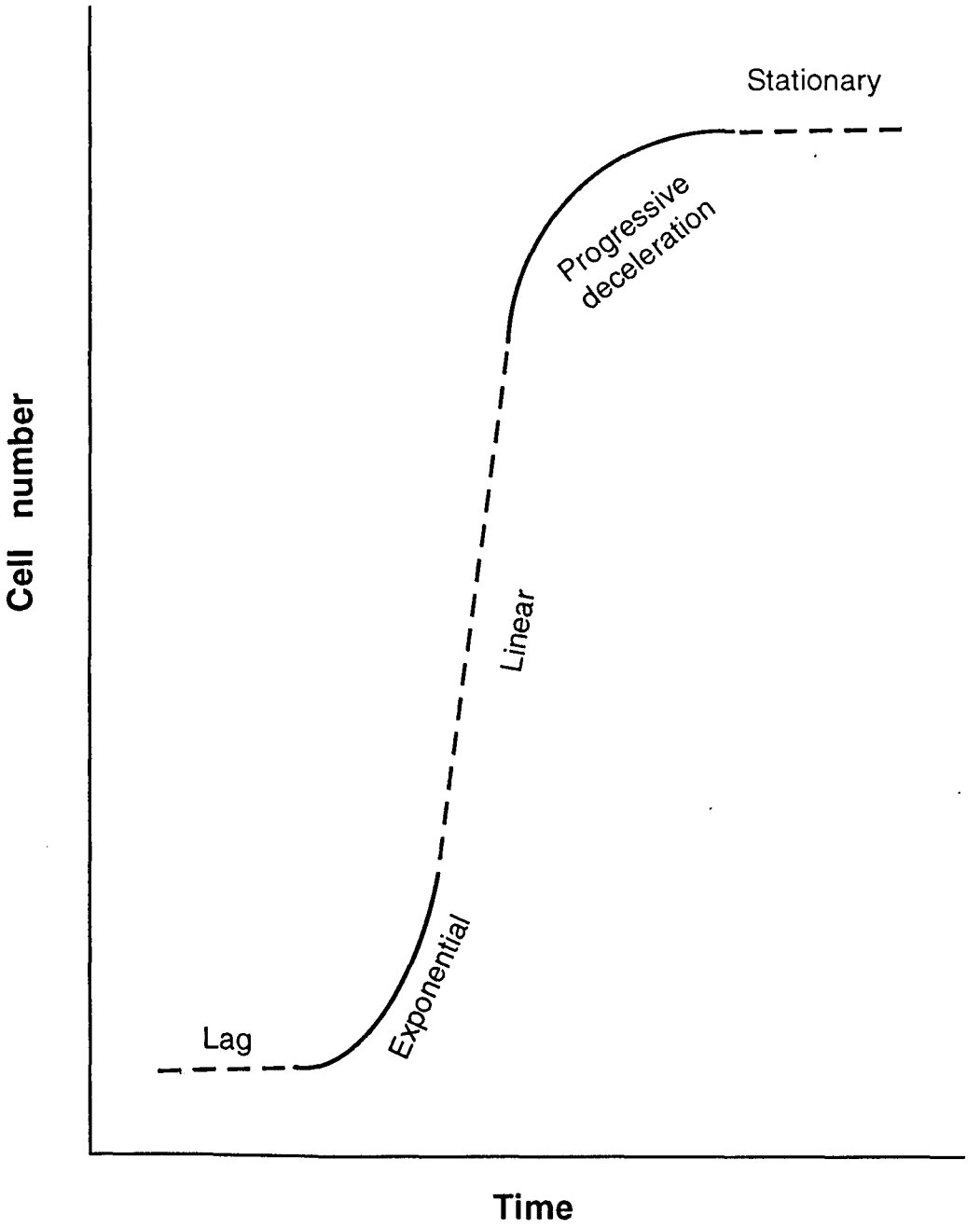


Fig. 1-7 Model curve relating cell number per unit volume of culture to time in a batch grown cell suspension culture

5. Stationary phase (cell numbers are constant).

During the lag phase the cells absorb water and nutritive substances and prepare for division. The volume of cytoplasm increases as do the numbers of mitochondria, ribosomes, endoplasmic reticulum and dictyosomes per cell. There is also a significant increase in both DNA and RNA content per cell (Short *et al.*, 1969).

During the exponential phase the cytoplasm appears more dense with the maximum content of endoplasmic reticulum and ribosomes. There are changes in respiratory activity (Givan and Collin, 1967) and many cells are in mitosis and cytokinesis. There is a decline in RNA levels probably due to both the catabolism of RNA and the decrease in RNA per cell due to cytokinesis (Short *et al.*, 1969).

Towards stationary phase, the cell aggregates begin to break up as cell division decelerates. The cells increase in size and become highly vacuolated with a thin layer of cytoplasm containing fewer organelles.

During the stationary phase there is an increase in starch deposition. The cells become senescent and eventually die (Henshaw *et al.*, 1966).

The growth pattern of cells in batch culture is very dependent on the initial inoculum density. A low inoculum density may result in a prolonged lag phase or possibly no growth at all. In some cases it may be necessary to monitor viability as incubation is continued beyond the beginning of the stationary phase.

1.6 Scope of the present study

The modification of plant part relationships so as to increase crop yields depends ultimately upon the regulation of cell division, enlargement and differentiation of the parts concerned (Elliott, 1982). Plant cell suspension cultures provide uniform systems in which the correlative influences present in the intact plant are removed and in which differential patterns of distribution of nutrients and hormones between the cells are essentially eliminated (Elliott *et al.*, 1982). Such systems are ideal for studies designed to discover precisely how cellular hormone level are regulated and in turn regulate cell division.

It has long been known that plant hormones play a vital role in the regulation of plant growth and development. However, there exist many factors that make contributions to the determination of the actual effect of the hormones. The ultimate effect of hormones is determined by their synthesis-degradation, inactivation, interconversion, transport and the sensitivity of tissues to them.

Following a series of investigations into the role of auxin in the regulation of cell division in suspension-cultured *Acer pseudoplatanus* L. cells (Moloney, 1979; Moloney *et al.*, 1983; Elliott *et al.*, 1987; O'Sullivan, 1988; Elliott *et al.*, 1988b, 1990), it is now generally accepted that hormone concentration and distribution within the cells together with receptor availability and distribution are determinants of the final effect of these hormones on cellular activities and plant growth. The NPA binding proteins are of special interest in these studies, since NPA appears specifically to block auxin efflux

by means of membrane bound binding sites (Rubery and Sheldrake, 1974; Rubery, 1979; Sussman and Goldsmith, 1981) and so stimulates net uptake by cultured cells, tissue segments and membrane vesicles. In the Department of Applied Biology and Biotechnology, Elliott *et al.* (1988b, 1990) have successfully demonstrated the importance of auxin transport and its control via NPA binding proteins (receptors) in the regulation of cell division. A strain of suspension cultured *Acer pseudoplatanus* cells which had been adapted to auxin-free medium had similar culture growth parameters to our 2,4-D requiring strain. The intracellular IAA concentration of the former cells was 1.15 ng 10^{-6} cells and of the latter 0.20 ng 10^{-6} cells. Both types of cells contained a high affinity membrane bound NPA binding protein with an estimated Kd of 7.5 x 10^{-9} mol dm^{-3}. The number of binding proteins (pmoles mg^{-1} membrane protein) changed through the batch culture cycle in a similar manner for both cell types but the 2,4-D free cells always contain 25-55% less of the NPA binding protein than the 2,4-D plus cells. Adaption to auxin-free medium seemed to involve a reduction in the capacity for carrier-mediated auxin efflux (Elliott *et al.*, 1988b, 1990). The first part of the present research programme has extended those studies on sycamore to the major commercial plant sugar beet, including characterization of the NPA binding proteins of that plant.

If we are to fully comprehend the regulatory systems governing plant development, it is essential that we determine the interaction between plant hormone levels, transport and signal transduction --- membrane and cytoplasmic receptor systems. The second part of this study will therefore concentrate on the characterization of the auxin binding proteins in sugar beet.

In the studies of auxin receptors, substantial progress has been made in the investigation of membrane-bound ABP-I. The first reports on binding were those by Hertel and co-workers (Lembi *et al.*, 1971; Hertel *et al.*, 1972). However, it took until 1985 before purification was achieved (Löbler and Klämbt, 1985a). With its purification to homology in maize and the consequent production of antibodies (Löbler and Klämbt, 1985a; Napier *et al.*, 1988), an elegent approach to identify its receptor function was initiated in Guern's group (Ephritikhine *et al.*, 1987). This was based on the fact that hyperpolarization was a characteristic auxin-induced response (Ephritikhine *et al.*, 1987; Shen *et al.*, 1988; Hager *et al.*, 1971, 1991). Series of work on using tobacco protoplasts in hyperpolarization study was published (Ephritikhine *et al.*, 1987; Shen *et al.*, 1988; Barbier-Brygoo *et al.*, 1989, 1990a,b,c). The hyperpolarization induced by auxin was inhibited by an antiserum to a plasma membrane H^+-ATPase (Barbier-Brygoo *et al.*, 1989), which showed that H^+ extrusion was involved. Addition of maize ABP antibody to protoplasts inhibited auxin-induced hyperpolarization (Barbier-Brygoo *et al.*, 1989). Important conclusions are suggested by this work. First, if antibody binding inhibits specifically auxin-induced responses, then ABP can be considered as a receptor. Second, the receptor population mediating hyperpolarization is on the outside of the plasma membrane and is coupled to plasma membrane H^+-ATPase activity. Thirdly, inhibition of hyperpolarization in tabacco protoplasts by the antibodies raised against maize ABP indicates that the receptor is highly conserved. The conclusion is further supported by using auxin-resistant mutants and *Agrobacterium rhizogenes* transformed plants (Barbier-Brygoo *et al.*, 1989, 1990a,b,c). It was found that more auxin-sensitive transformant had a greater number of plasma membrane receptors than the wild type and auxin-resistant mutant had fewer, consistent with receptor abundance on the plasma

membrane. Incubation of wild-type protoplasts with antibody caused a reduction in receptor population and the dose-response curve shifted to higher auxin concentration, while incubation with purified maize receptor resulted in an enhanced sensitivity. All these observations strongly support the conclusion that ABP is a receptor.

The purification of this receptor protein has also greatly facilitated the isolation and analysis of its gene. The determination of amino acid sequence of the purified protein allowed the synthesis of oligonucleotide probes for cDNA library screening. The sequence of the ABP-I gene was first published in 1989 (Hesse *et al.*, 1989; Inohara *et al.*, 1989). The increasing evidence of a receptor function for maize ABP, the success of receptor purification and gene isolation have opened the possibility to regulate auxin effects through the manipulation of the expression of its receptor gene(s). The second part of the programme involved the characterization, solubilization and purification of this protein in sugar beet. Continuation of this study will further our understanding of the properties of the protein, which could be of great importance in revealing the binding mechanism(s) of auxin to the receptors, and the immediate response of plant cells to this binding. In parellel, the action of ABP can also be studied by enhancing/supressing the expression of ABP gene(s). The initial steps undertaken in this study will be to detect and isolate the ABP gene(s) in sugar beet. These experiments have been greatly facilitated by the work of Löbler and Klämbt (1985a), Shimomuro *et al.* (1986), Napier *et al.* (1988), Hesse *et al.* (1989) and Inohara *et al.* (1989) who have purified the protein and determined the ABP gene sequence in maize. Complete maize ABP cDNA, which is available in the laboratory through the kind donation of Dr. C. M. Lazarus in the University of Bristol, will be used as a probe in the isolation of

ABP gene(s) in sugar beet, with the long-term objective that auxin effects in this plant could be regulated through its perception mechanisms by means of the manipulation of its receptor gene.

Chapter 2 Sugar Beet Cell Suspension Culture

2.1 Introduction

Plant cell suspension cultures provide a uniform system in which the correlative influences present in the intact plant are removed, and in which differential patterns of distribution of nutrients and hormones between cells are essentially eliminated (Elliott *et al.*, 1977). Cell suspension cultures are widely used as model systems for studying many aspects of cellular physiology and biochemistry (Dixon, 1985). The system is ideal for studies designed to discover precisely how cellular hormone levels are regulated and in turn regulate cell division and expansion, giving vital insights into the approaches required to regulate development *in vivo*. However, it is surprising, considering the economic importance of sugar beet, there is little published work on its tissue culture. In this laboratory, sugar beet root derived cell suspension cultures have previously been successfully used to identify the likely causal relationships between changing hormone profiles and developmental changes related to sugar accumulation (Elliott *et al.*, 1986). Since then, a variety of methods for initiating cell suspension cultures had been tested. However, the seedling root callus formed was very hard and compact and would not disperse in liquid medium to produce a cell suspension culture (Grieve, 1990).

In June 1986, a cell suspension culture was obtained from leaves of *Beta Vulgaris* cv Regina seedlings. The callus was developed on Murashige and Skoog (1962) solid medium

without added plant growth regulators. However, when placed into liquid culture it was supplemented with 1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} kinetin. The cell suspension cultures were transferred on a 21 day basis and growth parameters were measured (Ryan, 1988; Grieve, 1990). However, when either kinetin or 2,4-D was withheld from some of the subcultured suspensions, the cells continued to grow and the growth parameters were seen to be similar to the cells grown on medium containing 2,4-D and kinetin. Therefore, questions arose as to the following two points:

(1) whether the exogenous plant growth regulators are required for sugar beet seedling leaf derived suspension cultures?

(2) Whether future study on auxin economy could be based on the comparison of auxin-dependent and auxin-independent cell strains?

Meanwhile, it is also important when embarking on further investigations to ensure that the strain of cells under study still exhibit the same basic patterns of growth.

2.2 Materials and methods

2.2.1 Chemicals

All chemicals used were analytical grade reagents from BDH (Poole, Dorset), Sigma

(Poole, Dorset), Pharmacia (Sweden), Fisons (Loughborough, Leicestershire) and Phase Sep (Deeside, Clwyd).

2.2.2 Glassware

All glassware used was Pyrex. New glassware was filled with distilled water and autoclaved at 121°C for 20 min prior to use as culture apparatus. Routine cleaning of glassware involved soaking overnight in Decon 90 (BDH) 50 cm^3 dm^{-3}, followed by vigorous brushing, three times rinsed three times with tap water and finally rinsed three times with deionized water.

2.2.3 Sterilisation

The basic procedures for maintaining sterile cultures have been described by Henshaw *et al.*, 1966) and Street (1973). All media were sterilised by autoclaving for 20 min at 121°C (100KPa). All aseptic transfers were performed in a sterile room fitted with a twin Laminair flow unit (Pathfinder) using instruments that had been sterilised either by autoclaving or by flaming in alcohol. Flasks were sealed with a double layer of autoclaved aluminium foil (Alcan).

2.2.4 Cell suspension cultures

Sugar beet (*Beta vulgaris* L.) cell suspension cultures from leaves of Regina seedlings were produced from the callus which developed on Murashige and Skoog (1962) medium without added plant growth regulators (Table 2-1). However, when placed into liquid culture, it was supplemented with 1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} kinetin. The stock cultures were grown in 250 cm^3 narrow necked Erlenmeyer flasks (20 cm^3 inoculum and 80 cm^3 medium) and subcultured every 21 days. In the later part of this programme, the cells were subcultured every 16 days (see section 2.4).

All fixed-volume additions under aseptic conditions were performed by the use of an automatic pipetting unit (A.R. Horwell Ltd) set to the appropriate volume and autoclaved under the same conditions as the medium. Wide bore cannulae (3 mm) were used to pipette cell suspensions. For liquids, medium-bore cannulae were employed in order to reduce dead volumes to a minimum.

Where larger amounts of material were required, cells were grown in 1 dm^3 bottles containing 500 cm^3 of culture. This comprised 400 cm^3 of culture medium and 100 cm^3 of stationary phase (day 16) culture, i.e. a single stock flask.

All the culture flasks were incubated in the light (500 $cd.sr.m^{-2}$ at 25 °C on flat bed shakers (L.H. Engineering Co. Ltd.) at 120 cycles per minute as advised by Rajasekhar *et al.* (1971). Batch culture propagation was used in this study.

Table 2-1 Murashige and Skoog medium

(Murashige and Skoog, 1962)

	Concentration of stock solution (g dm^{-3})	Volume of stock solution per dm^3 of medium (cm^3)
A. Macronutrients		
NH_4NO_3	33	
KNO_3	38	
$CaCl_2 \cdot 2H_2O$	8.8	50
$MgSO_4 \cdot 7H_2O$	7.4	
KH_2PO_4	3.4	
B. Micronutrients		
KI	0.166	
H_3BO_3	1.24	
$MnSO_4 \cdot 4H_2O$	4.46	
$ZnSO_4 \cdot 7H_2O$	1.72	5
$Na_2MoO_4 \cdot 2H_2O$	0.05	
$CuSO_4 \cdot 5H_2O$	0.005	
$CoCl_2 \cdot 6H_2O$	0.005	
C. Iron source		
$NaFe(EDTA)_2$	2.67	10
Meso-inositol	10	
D. Vitamins		
Nicotinic acid	0.1	
Pyridoxine-HCl	0.1	5
Thiamine-HCl	0.1	
Glycine	0.4	
E. Carbon source		
Sucrose		30 g dm^{-3}
Adjusted to pH 5.6-5.8, using 1.0 mol dm^{-3} KOH		

2.2.5 Sterility

Cultures were regularly checked for contamination by plating out samples onto 15 g dm^{-3} nutrient agar. Beside nutrient agar, cell cultures were also plated onto the following media: Corn meal agar; Czapek dox agar; Potato dextrose agar and malt extract agar. This range of media were chosen to detect the possibility of a range of contaminants. All these plates were incubated at 37°C for two weeks, or at 25°C for three weeks.

2.2.6 Cell viability

5 cm^3 cell culture samples were taken, 1 cm^3 of a phenosafranin solution (1 g dm^{-3}) was added to each and the mixtures were agitated for 5 min using a Whirlimixer. After standing at room temperature for 5 min the samples were centrifuged for 5 min at 2,000 *g* in a Gallenkamp bench centrifuge. The supernatant was discarded and the cells were then washed with 5 x 5 cm^3 distilled water and a sample was placed on a microscope slide. The dead cells are stained red by phenosafranin. The total cell number and the number of stained cells were recorded for five fields and the percentage of non-viable cells was estimated.

2.2.7 Growth parameters

The methods for determining growth parameters were described by Henshaw *et al.* (1966).

2.2.7.1. Packed cell volume (PCV)

PCV was determined by transfer of 5 cm^3 of culture to a 10 cm^3 graduated centrifuge tube and centrifugation in a Gallenkamp bench centrifuge for 5 minutes at 2,000·g. The PCV was expressed as volume of cell pellet per unit volume of culture.

2.2.7.2 Fresh mass and dry mass

After measuring PCV, the cells were washed free of medium and resuspended in the same volume of distilled water. The fresh mass was measured by filtration through a previously weighed glass fibre filter (Whatman GF/A) on a multifiltration unit (Strand Scientific). The samples were then oven-dried (minimum 8 hrs) to a constant weight at 80°C to obtain the dry mass.

2.2.7.3 Cell counting

5 cm^3 of culture samples were freed of medium and washed with distilled water before being resuspended in 10% (w/v) chromium trioxide solution. The samples were then stored at 4°C for no longer than 14 days or counted immediately. To facilitate counting, the samples were incubated in a water bath at 70°C for 20-40 minutes and then agitated using a Whirlimixer for 5 minutes to disperse the cell aggregates. The samples were diluted with

distilled H_2O to a known volume and a few drops of the homogenous samples were placed on a recessed microscope counting slide (1 mm deep). Cell numbers were determined by counting 10 fields 5 times under a Vickers microscope with a magnification of x 100. Cell numbers per cm^3 were calculated from the following formula:

$$N = \frac{X\ D}{K}$$

where N = cell number x 10^{-3} per cm^3

X = average number of cells per field

D = dilution factor

K = constant for the microscope and slide used, accounting for the volume of the field

2.3 Results

2.3.1 Growth parameters of sugar beet seedling leaf cell suspension culture

Sugar beet seedling leaf derived cell suspension cultures supplied with 2,4-D and kinetin have been maintained in this laboratory since 1986. The culture conditions have been detailed in section 2.2.4, subculture being carried out every 21 days. The growth parameters were estimated as described in section 2.2.7.

The changes of the growth parameters during batch culture growth used in this study are represented graphically in Fig. 2-1 to 2-3. Packed cell volume (PCV) is expressed as a percentage and all the error bars show standard deviation of the mean of three samples.

2.3.2 Estimation of the effect of plant growth regulators on growth parameters for sugar beet seedling leaf cell suspension cultures

The comparisons on the growth parameters were carried out on sugar beet seedling leaf cell suspension cultures supplied with 2,4-D and kinetin, kinetin only, or in the absence of plant growth regulators. Both 2,4-D-free and PGR-free growth data presented here are for the third passage after 2,4-D or PGRs were omitted. The suspension cultures supplied with 2,4-D and kinetin have been transferred for eight passages on a 21 day basis since the last measurement (section 2.3.1).

These cell lines were monitored by PCV, fresh mass, dry mass and cell number. The comparisons of the growth parameters are represented graphically in Fig. 2-4 to 2-7.

It can be seen that the growth data for the cells with 2,4-D and kinetin, without 2,4-D and without both PGRs had no significant differences during the third passage following withdrawal of 2,4-D or both PGRs. When the cells supplied with 2,4-D and kinetin were directly transferred to PGR-free medium, the cells continued to grow and the growth parameter data for the cells did not show any significant difference. Meanwhile, the

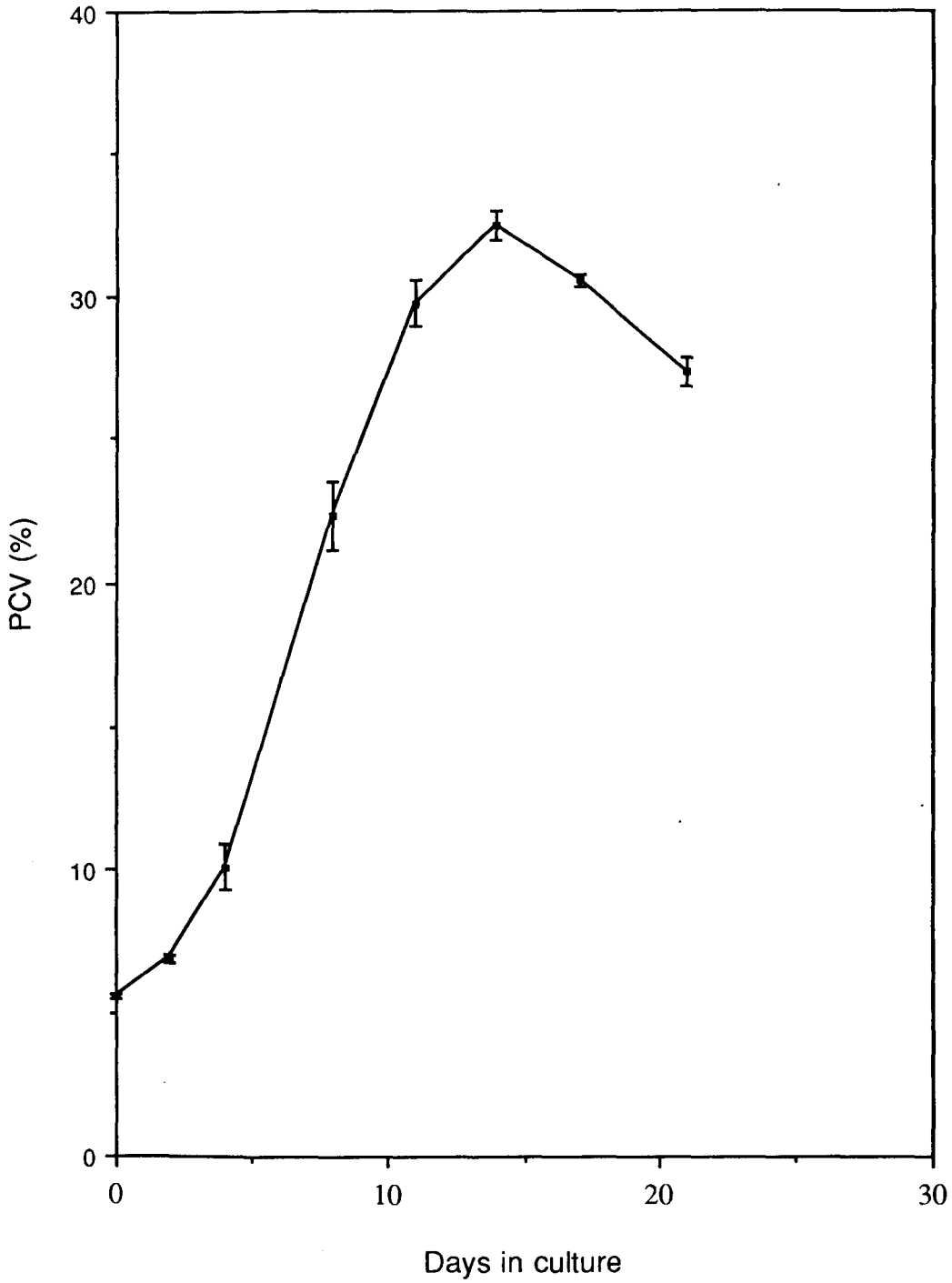


Fig. 2-1 Changes in PCV of B.vulgaris cells during batch culture growth

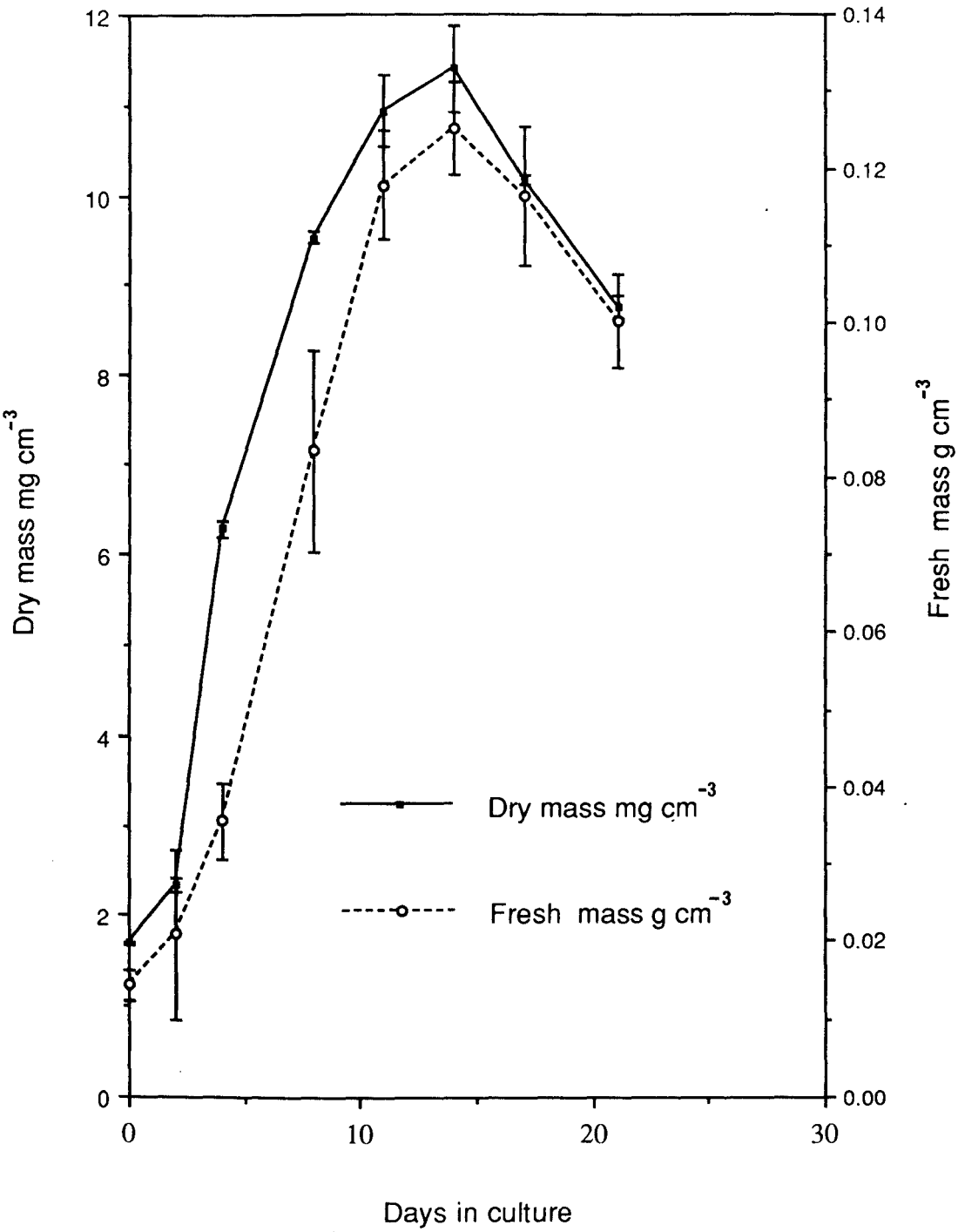


Fig. 2-2 Changes in fresh mass and dry mass of B. vulgaris cells during batch culture growth

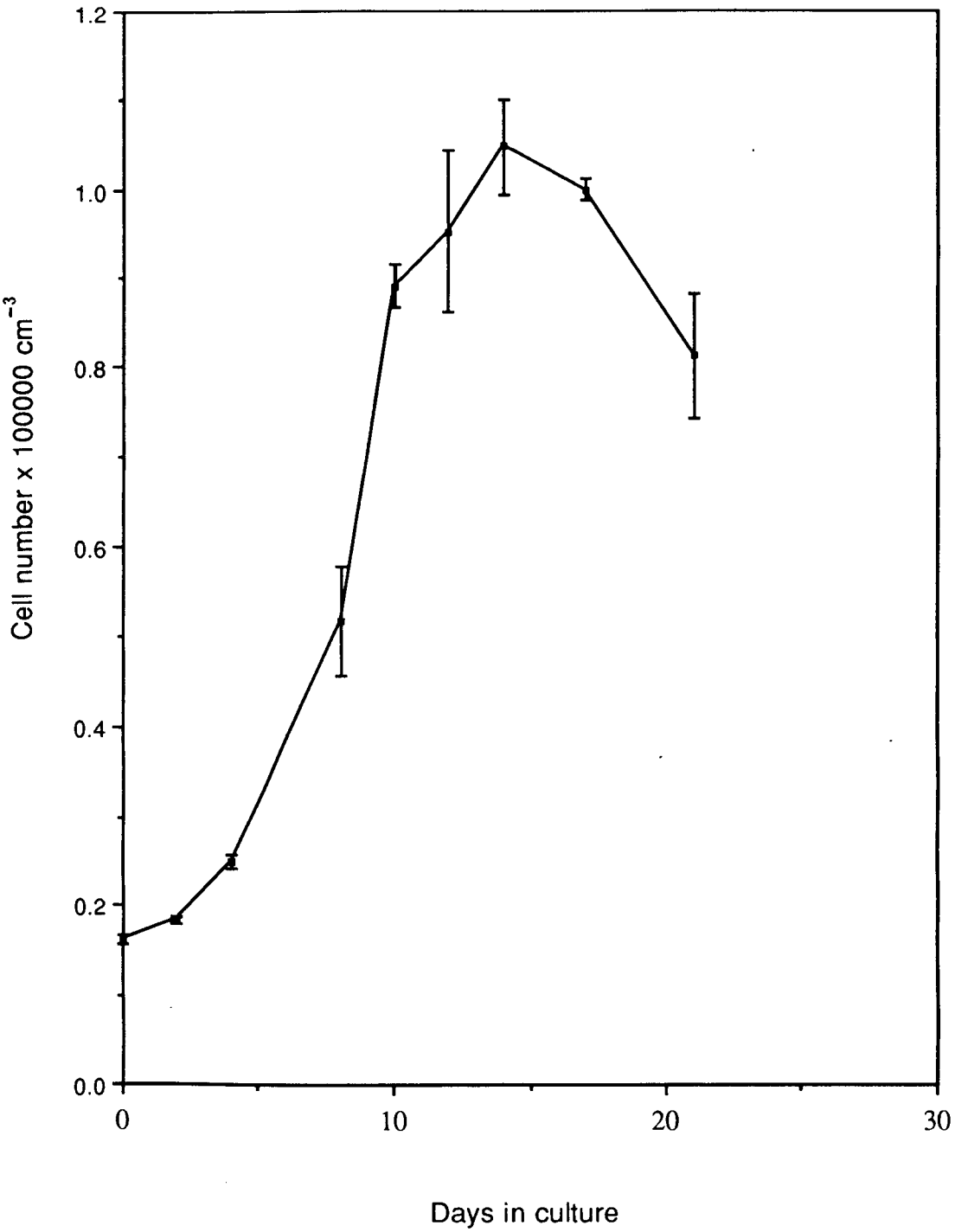


Fig. 2-3 Changes in cell number of B. vulgaris cells during batch culture growth

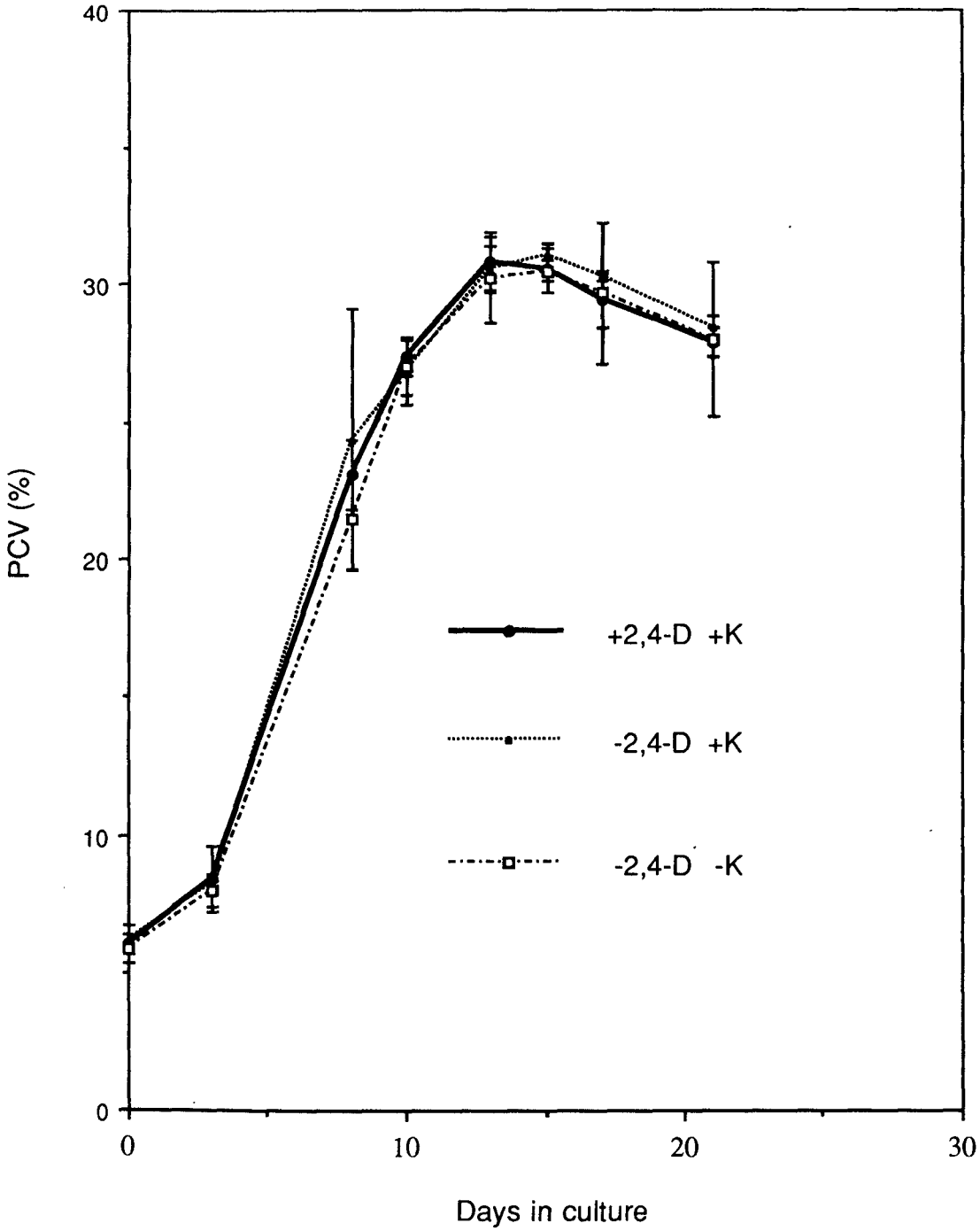


Fig. 2-4 Effect of plant growth regulators on PCV of B. vulgaris cells during batch culture growth

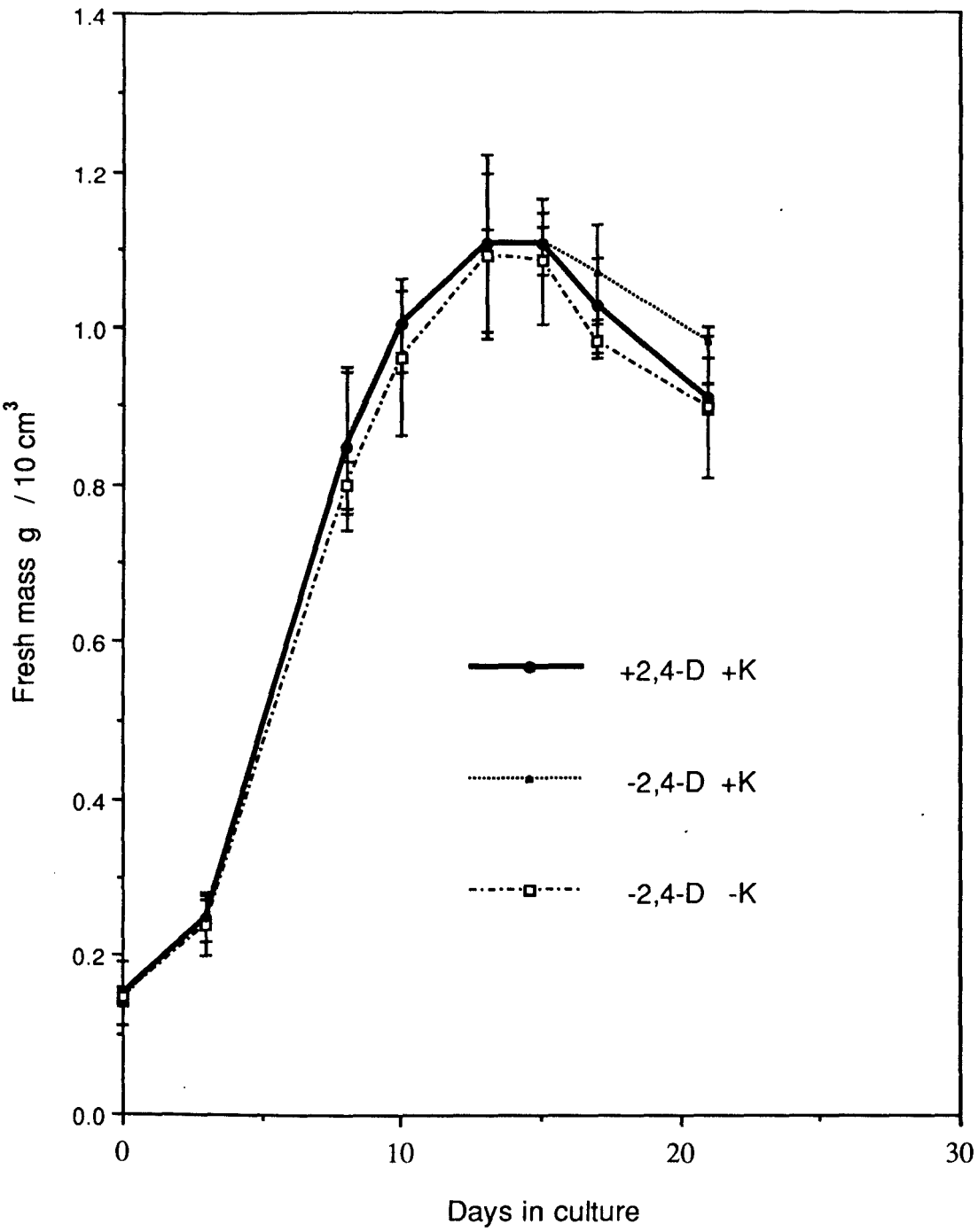


Fig. 2-5 Effect of plant growth regulators on fresh mass of B. vulgaris cells during batch culture growth

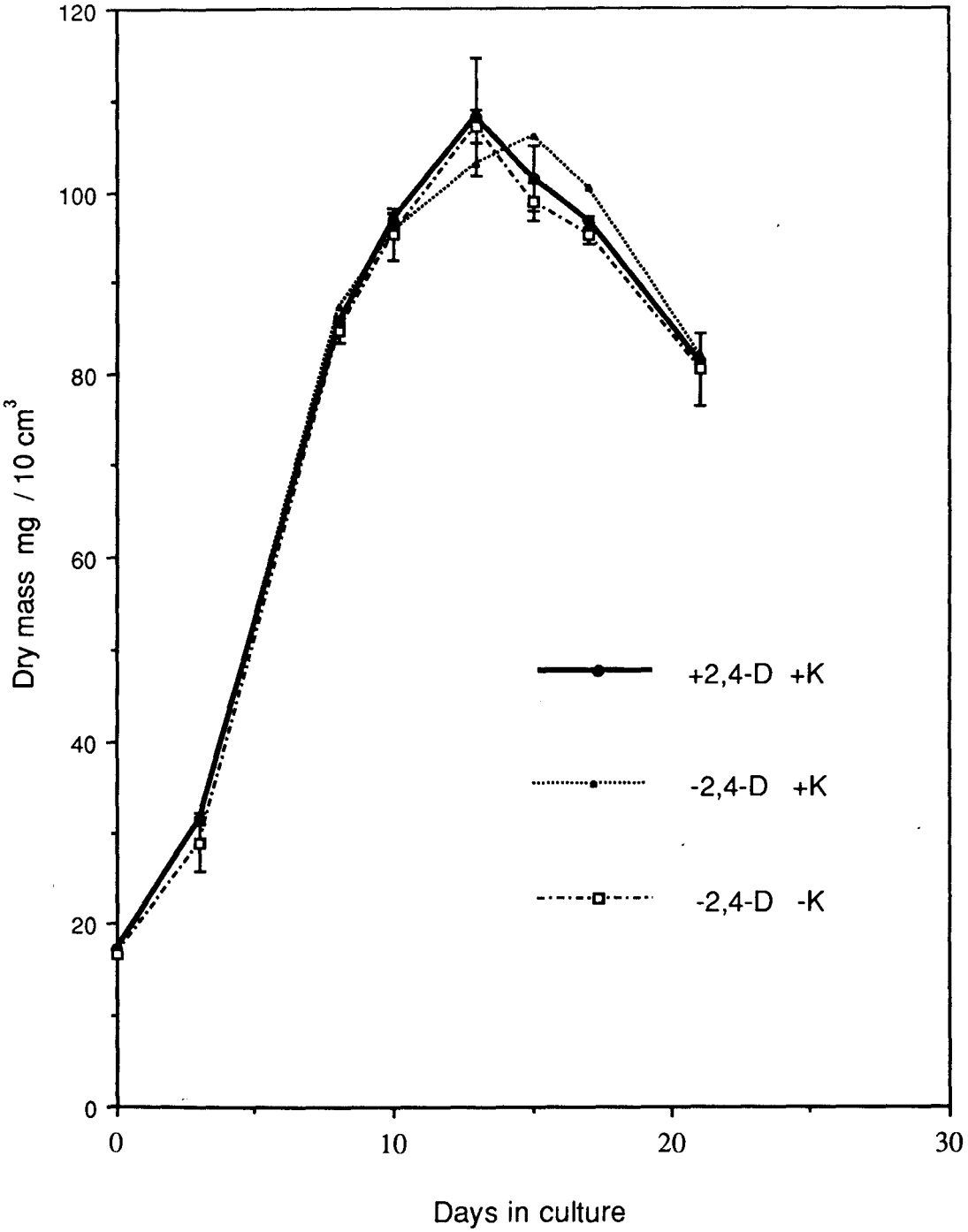


Fig. 2-6 Effect of plant growth regulators on dry mass of B. vulgaris cells during batch culture growth

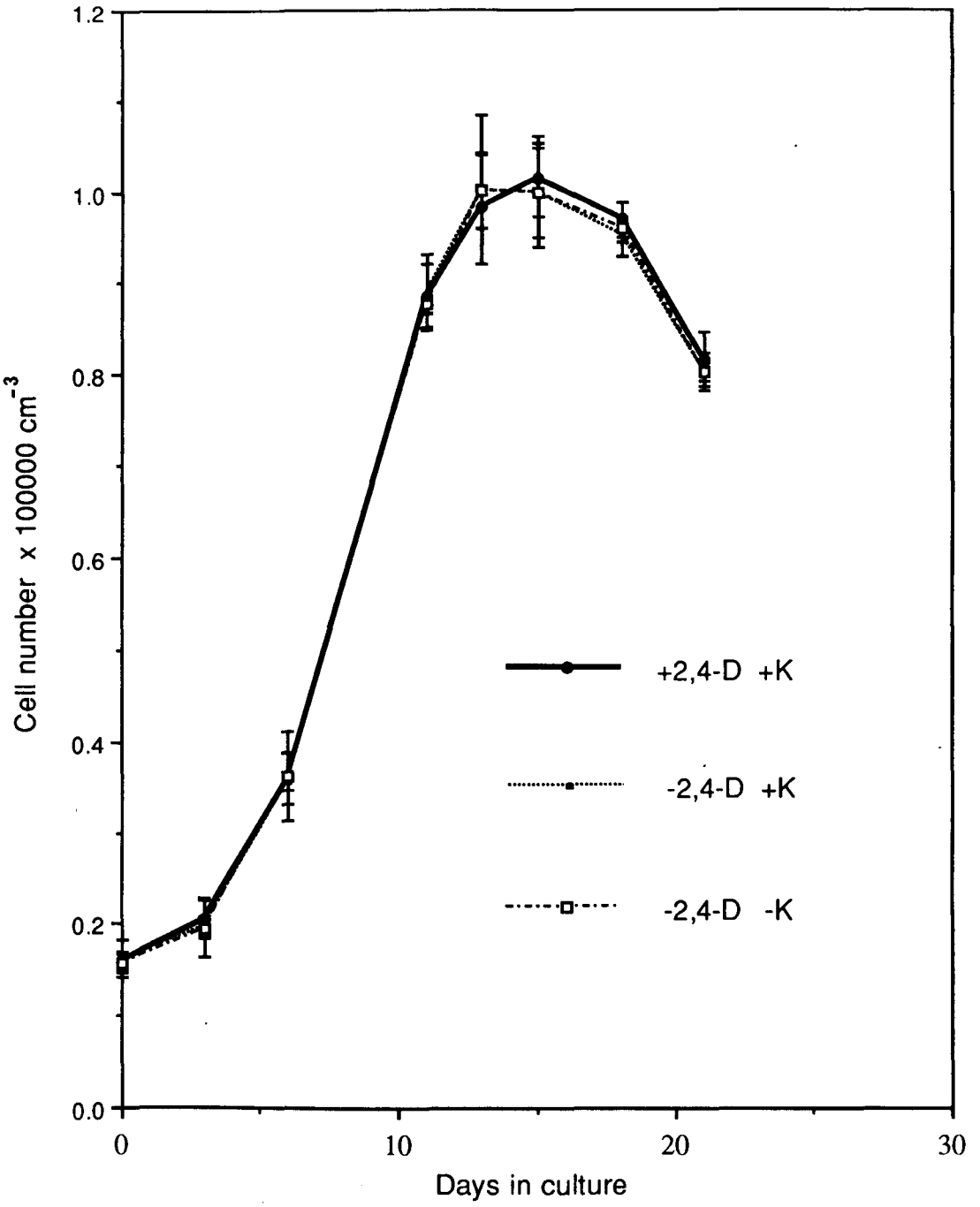


Fig. 2-7 Effect of plant growth regulators on cell number of B. vulgaris cells during batch culture growth

culture in which only 2,4-D was withheld also had similar characteristics to the one supplemented with 2,4-D.

This indicates that the sugar beet seedling leaf derived cell suspension cultures are indeed hormone-autonomous. 2,4-D and kinetin are not necessary for cell growth and these are not an auxin-requiring cell line for sugar beet seedling leaf cell suspension cultures.

2.4 Discussion

In this chapter the growth parameters of sugar beet seedling leaf derived cell suspension cultures were investigated. The majority of the information available to date on the growth and metabolism of cultured cells has been obtained from batch suspension cultures.

Plant cells in batch culture, i.e. cultures in a fixed volume of culture medium, increase in biomass by cell division and cell growth until a factor in the culture environment becomes limiting and sends them into a stationary phase (Wilson *et al.*, 1971). This may be due to a limiting factor such as sucrose, nutrients or oxygen level, or possibly due to a build-up of a toxic substance. At this stage of growth, a sterile sample of the culture must be taken to inoculate into fresh medium, otherwise the culture will senesce and eventually die. On the other hand, the growth pattern of cells in batch culture is very dependent on the initial inoculum density. A low inoculum density may result in a prolonged lag plase or possibly no growth at all. Growth may become imbalanced in cultures resulting in the loss of the

coupling between biosynthesis and the cell cycle (Henshaw *et al.*, 1966).

Sugar beet seedling leaf cell suspension cultures were initially transferred on a 21 day basis. However, it can be seen from the growth parameters that the cell density reached a maximum around day 14 and then it started to decline. A sharp decrease was seen, especially from day 18 of growth. Cell viability (Widholm, 1972) in cultures with and without plant growth regulators at day 18 and day 21 was compared in Table 2.2. There is a significant decrease in viable number from day 18 to day 21. A similar result was found by colleagues (Ryan, 1988; Fowler, personal communication). This demonstrates that cell viability cannot be maintained in stationary phase cultures for extended periods (>3-4 days). Thus, it is essential that the sterile samples of the culture should be taken to inoculate into fresh medium between day 16 and day 18, rather than at day 21.

Table 2.2 Percentage of the cell viability in cultures*

Cultures	Days	
	18	21
+ 2,4-D + kinetin	95.25±2.21	82.46±0.64
- 2,4-D - kinetin	93.88±1.07	80.10±1.56

*The error bars show standard deviation of the mean of four replicates.

It has been noted that plant cell cultures often required an auxin in their culture medium if cell division and enlargement were to occur (Gautheret, 1955). When *Acer*

pseudoplatanus L. cells were transferred from the standard (2,4-D containing) culture medium to medium lacking this auxin, there was a decline in the rate of cell division giving a reduced final cell number and cell lysis often occurred (Street *et al.*, 1968; King, 1976). In comparison however, suspension-cultured sugar beet seedling leaf cells, by the third passage in 2,4-D free medium, continued to grow and there were no significant differences in growth compared to those grown in 2,4-D containing medium. Such results were different from those found in *Acer pseudoplatanus* L. cells. *Acer pseudoplatanus* L. cells could not be transferred directly into medium without 2,4-D (Elliott *et al.*, 1988b). The culture which did not require 2,4-D for growth (NDR) was derived from standard cells (DR) by transfer to medium containing an increased level (x 5) of nitrate, in which potassium ions replaced sodium ions. After three passages of 21 days following withdrawal of 2,4-D from standard medium, such cells had similar growth parameters to those of containing 2,4-D in the medium (Hall, 1979). When intracellular IAA levels of *Acer pseudoplatanus* L. cells were investigated between DR and NDR lines, it was found that IAA concentration for the NDR cell line was approximately six times higher than DR cell line (Elliott *et al.*, 1988b). However, the concentrations of intracellular IAA levels for sugar beet seedling leaf cells grown with and without 2,4-D were similar (Ryan, 1988). This evidence indicates that sugar beet seedling leaf cell suspension cultures are indeed auxin-autonomous.

When sugar beet seedling leaf cell suspension cultures were transferred to PGR-free medium after three passages, the cells had similar growth parameters to cells in medium

with plant growth regulators. It had been noted that sugar beet seedling leaf cell suspension cultures were obtained from the leaf callus which developed on Murashige and Skoog (1962) solid medium without added plant growth regulators. The production of such hormone-autonomous callus was recently supported (Masuda *et al.*, 1988). Similar instances with tobacco and soybean were reported as cytokinin-autonomous (Miura and Miller, 1969; Dyson and Hall, 1972; Einset and Skoog, 1973). The growth parameters of sugar beet seedling leaf cell suspension cultures were monitored for the first three passages following withdrawal of kinetin, it was found that such cultures were indeed cytokinin-autonomous (Grieve, 1990).

DeGreef and Jacobs (1979) noted that the sugar beet callus from seedling leaf tissue after habituation treatment could be maintained on PGR-free medium. Therefore, this would imply that sugar beet seedling leaf derived cell suspension cultures are hormone-autonomous due to a sufficient level of endogenous hormone for cell division.

Since sugar beet seedling leaf derived cell suspension cultures were shown to be hormone-autonomous, a good comparable system, i.e. auxin-dependent and auxin-independent cell lines, cannot be established. This means that there is no possibility to investigate auxin-related biochemical and molecular biological mechanisms through changing the availability and status of exogenous auxin with this cell line. However, such a culture was considered to be advantageous as it rendered the investigation of the determinants and their interactions in auxin economy easier due to the absence of any

additional auxins needed to support cell growth.

Chapter 3 The Role of Phosphorylation in Control of NPA Binding Protein Activity

3.1 Introduction

The first convincing plant growth regulator binding protein which exhibited receptor-like features was detected in maize (*Zea mays* L.) plasma membranes for the synthetic auxin transport inhibitor N-1-naphthylphthalamic acid (NPA).

The existence of NPA binding proteins has been demonstrated in maize, tobacco, pea, English sycamore and other plants (Lembi *et al.*, 1971; Thomson *et al.*, 1973; Trillmich and Michalke, 1979; Normand *et al.*, 1975; Katekar *et al.*, 1981; Maan *et al.*, 1985; Elliott *et al.*, 1988b). According to the chemiosmotic model of auxin transport, polarity of transport is achieved by efflux of auxin anions from the cell via specific, saturable auxin efflux carriers that are located in the plasma membrane preferentially at the basal ends of auxin transporting cells (Rubery and Sheldrake, 1974). NPA is thought to act by blocking the auxin efflux carrier and therefore NPA binding should be a marker for this carrier. Jacobs and Gilbert (1983) raised monoclonal antibodies against the NPA binding protein by immunisation with pea membranes, using inhibition of NPA binding as the screen. Immunofluorescence studies showed that the antibodies specifically labelled U-shaped regions at the basal ends of transporting vascular parenchyma cells, as required by the chemiosmotic hypothesis.

The NPA binding protein has been solubilized from the membranes (Sussman and Gardner, 1980; Thein and Michalke, 1988) and partially purified (Jacobs and Short, 1986). The most significant change in properties upon solubilization was the ability of IAA (10^{-6}-10^{-5} mol dm^{-3}) to displace ^{3}H-NPA from the binding proteins, whereas with native ones, in agreement with other reports on maize and other species (Maam *et al.*, 1985; Elliott *et al.*, 1990), no interaction with IAA was observed even at 0.5 mmol dm^{-3} (in maize). The affinity for α-NAA and ß-NAA was also enhanced by solubilization, by at least an order of magnitude. This report was the first indication that the NPA binding protein complex is able to interact to some degree with IAA.

Characteristics and possible functions of NPA binding sites have been successfully examined in suspension cultured *Acer pseudoplatanus* L. cells in this laboratory (Elliott *et al.*, 1988b, 1990). These cells normally require 2,4-D as an exogenous auxin for cell division. Transferring these "2,4-D requiring (DR)" cells into 2,4-D free medium caused reduction in rate of cell division. A "no 2,4-D requiring (NDR)" strain has been adapted to auxin-free medium and has similar culture growth parameters to DR strain cells. The intracellular IAA concentrations of DR cells and NDR cells were 0.20 and 1.15 ng 10^{-6} cells (at day-7 in the batch culture cycle). It was found that both cell strains contained a high affinity membrane-bound NPA binding protein with Kd of 7.5 x 10^{-9} mol dm^{-3}. The number of binding proteins changed through the batch culture cycle in both strains but NDR strain always contained less amount than DR strain cells (pmoles mg^{-1} membrane protein). Habituation of the NDR strain to auxin-free medium seemed to involve a

reduction in the capacity for carrier-mediated auxin efflux. These studies have demonstrated that the NPA binding protein localized in the plasmalemma played a crucial part in establishing levels of intracellular IAA appropriate for cell division.

It is known in animal systems that the biological activity of membrane receptors is highly regulated and that this regulation plays a major role in modulating the sensitivity of cells to extracellular signals. Through intensive studies in animal systems, it is now clear that protein phosphorylation of receptors is the primary mechanism of regulation of receptor function (Huganir and Greengard, 1987). However, studies on phosphorylation of plant hormone receptors have been very limited.

The objective of this study is to characterise the NPA binding proteins in sugar beet seedling leaf cell suspension culture. Special attention will be given to its possible regulation by phosphorylation.

3.2 Material and methods

3.2.1 Plant materials

Sugar beet (*Beta vulgaris* L.) cell suspension cultures from leaves of *cv.* Regina seedlings were produced from the callus which developed on Murashige and Skoog (1962) medium without added plant growth regulators. The stock cultures were grown in 250 cm^3 narrow

necked Erlenmeyer flasks (20 cm^3 inoculum and 80 cm^3 medium) and subcultured every 16 days. All the culture flasks were incubated in the light (500 $cd.sr.m^{-2}$) at 25°C on flat bed reciprocating shakers (L. H. Engineering Co. Ltd) at 120 cycles per minute as recommended (Rajasekhar *et al.*, 1971). Batch culture propagation was used in this study.

3.2.2 Membrane isolation

Techniques used for NPA binding assay were similar to those described previously (Elliott *et al.*, 1990; Maan *et al.*, 1985). Cells (day-5 to day-10 unless indicated) from suspension cultures were harvested by filtering under reduced pressure and rinsed twice with ice cold distilled water. The tissue was homogenised with pestle and mortar in an equal volume of isolation buffer (50 mmol dm^{-3} Tris-base, 1 mmol dm^{-3} Na_2EDTA, 0.1 mmol dm^{-3} $MgCl_2$ and 0.25 mol dm^{-3} sucrose, adjusted to pH 8.0 with KOH) for 5-6 minutes. The homogenate was centrifuged at 4,000 *g* for 10 minutes. The pellets were discarded and the supernatant was centrifuged at 50,000 *g* for 45 minutes. The pellet was then resuspended in assay buffer (50 mmol dm^{-3} Na_3 citrate/citric acid, 5 mmol dm^{-3} $MgCl_2$, 0.25 mol cm^{-3} sucrose, pH 4.5) at a concentration of 2 g initial fresh weight per cm^3. The whole isolation procedure took place at 4°C.

3.2.3 Binding assays

3.2.3.1 Binding affinity

The membrane suspension (0.5 cm^3) was incubated with a fixed amount ($4x10^{-10}$ mol dm^{-3} final concentration) of ^{3}H-NPA (specific activity 1.81 TBq $mmol^{-1}$, Amersham) and different concentrations of unlabelled NPA, from 0 to 10^{-4} mol dm^{-3} in polycarbonate tubes giving a final assay volume of 2.5 cm^3. The samples were incubated with shaking for 60 minutes at 4°C and then centrifuged at 75,000 *g* for 30 minutes. The supernatants were decanted, the sides of the tubes were carefully surface-washed twice with assay buffer (being careful not to touch the pellet) and the tubes were inverted for about 10 minutes to drain. Finally, the tube walls were dried with a roll of chromatography paper to diminish considerably carry-over of radioactivity by the tube walls. One cm^3 ethanol was placed in each tube. After 16 hours at room temperature or at least 30 minutes at 60°C, which was sufficient to extract all radioactivity from the pellet, the ethanol together with the pellets were transferred to scintillation vials for counting.

3.2.3.2 Association and dissociation

Binding assays, using a filtration technique, were based on that of Trillmich and Michalke (1979). The total binding was determined by incubating the membrane suspension with $4x10^{-10}$ mol dm^{-3} ^{3}H-NPA, and the non-specific binding was determined by incubating the membrane suspension with both $4x10^{-10}$ mol dm^{-3} ^{3}H-NPA and 10^{-4} mol dm^{-3} unlabelled NPA at 4°C. Triplicate samples were taken at regular intervals and filtered immediately through Whatman GF/C filters (Springfield Mill, U.K.). Usually the filter was washed with an additional 10 cm^3 of ice-cold assay buffer, which had been diluted 5 times before

use. When all the liquid had filtered through, the vacuum was shut off. The filtration and washing must be completed in less than 30 seconds to minimize the dissociation during the washing. After incubation for 60 min, unlabelled NPA was added giving a final concentration of 10^{-4} mol dm^{-3}. The same procedure as described above was followed. The "filter background" using just radioactive medium of the same concentration as in the assay, but without the membrane suspension, was determined and subtracted.

3.2.3.3 Competition experiments

The membrane suspension and 4 x 10^{-10} mol dm^{-3} ^{3}H-NPA were incubated with different concentrations of unlabelled competitors. Bound and free NPA were separated by the centrifugation method described above. The Kd was determined as the concentration of competitor which displaced 50% of the labelled NPA from the binding sites.

3.2.3.4 Binding assays in the presence of Mg^{2+} ATP or acid phosphatase

The membrane suspension was divided into two equal portions. In one portion, 0.5 cm^3 of 25 mmol dm^{-3} Mg^{2+} ATP or 0.5 cm^3 buffer containing 1 unit acid phosphatase was added to the binding assay mixture, giving a final asay volume of 2.5 cm^3, unless otherwise indicated, while the other was run as a control. Two point binding assays were carried out as described above, but two fixed concentrations of unlabelled NPA, 10^{-8} mol dm^{-3} (for total binding) and 10^{-4} mol dm^{-3} (for non-specific binding) were used (together with 4 x 10^{-}

^{10} mol dm^{-3} ^{3}H-NPA) as a relative measure for specific NPA binding.

3.2.4 Determination of endogenous phosphatase activity

An aliquot of 0.1 cm^3 supernatant was added to 0.9 cm^3 p-nitrophenyl phosphate (pNPP) solution dissolved in isolation buffer (pH 8.0). Hydrolysis of pNPP was measured over a minute period. The endogenous phosphatase activity in the sample was determined from the absorbance change at 410 nm.

3.2.5 Protein quantification

Protein contents of membrane suspensions were initially determined according to Bradford (1976). Later the modification of Read and Northcote (1981) was followed.

3.3 Results

3.3.1 pH optimum

3.3.1.1 Effect of pH of isolation buffer on specific binding of ^{3}H-NPA

The fresh mass of sugar beet seedling leaf cell suspension culture was divided into five equal parts. Each part was homogenised in isolation buffer ranging from pH 3.0 to pH

8.0. After the first high-speed centrifugation (i.e. 50,000 *g* for 45 min), pellets extracted with isolation buffers at different pHs were resuspended separately in pH 4.0 assay buffer and were used in a binding assay. The result is given in Fig. 3-1. Each column shows the mean of three measurements. It can be seen that the maximum specific binding was obtained when membrane pellets were isolated at pH 8.0. Thus, the optimal pH in the test for the extraction of the binding site-containing membrane pellet appeared to be alkaline, around pH 8.0.

3.3.1.2 Effect of pH of assay buffer on specific binding of ^{3}H-NPA

The cells from sugar beet seeding leaf suspension cultures were homogenised in isolation buffer at pH 8.0. After the bench centrifugation, the supernatant was divided into five equal parts for high speed centrifugation. The pellets obtained were separately resuspended in assay buffers of different pH ranging from pH 3.0 to 5.0. Fig. 3-2 shows the effect of the pH of the assay buffer on specific ^{3}H-NPA binding. It can be seen that there is a distinct optimum pH at 4.5. The results show the mean value of three replicate samples and the assay has been repeated twice. Similar results were obtained by the filtration method. Therefore, the optimal pH of assay buffer for specific NPA binding was pH 4.5. This pH was used in the binding assays throughout the study in sugar beet.

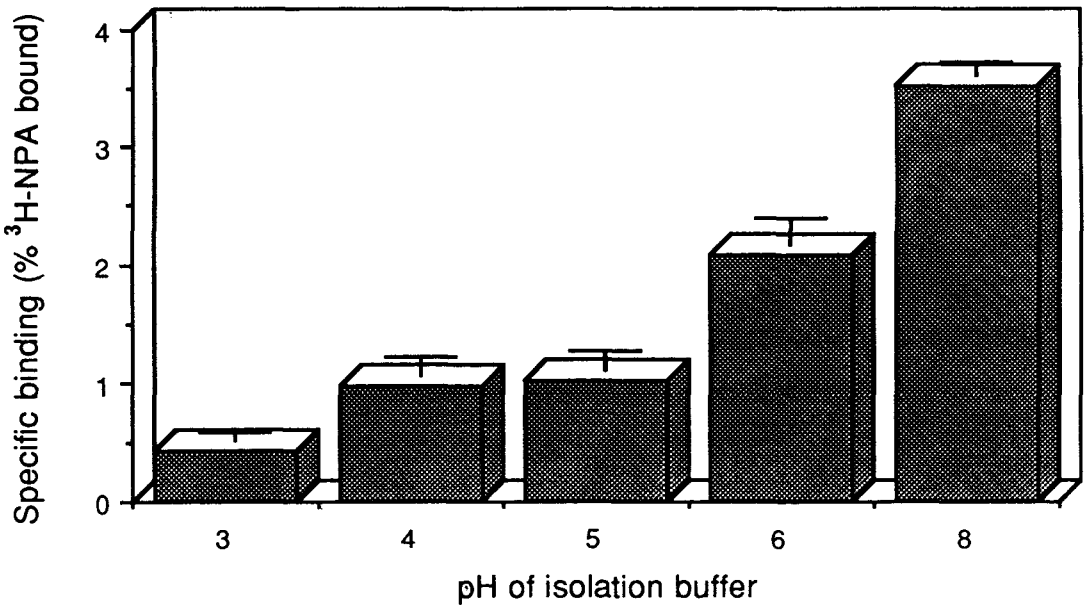


Fig. 3-1 Effect of pH of isolation buffer on specific NPA binding

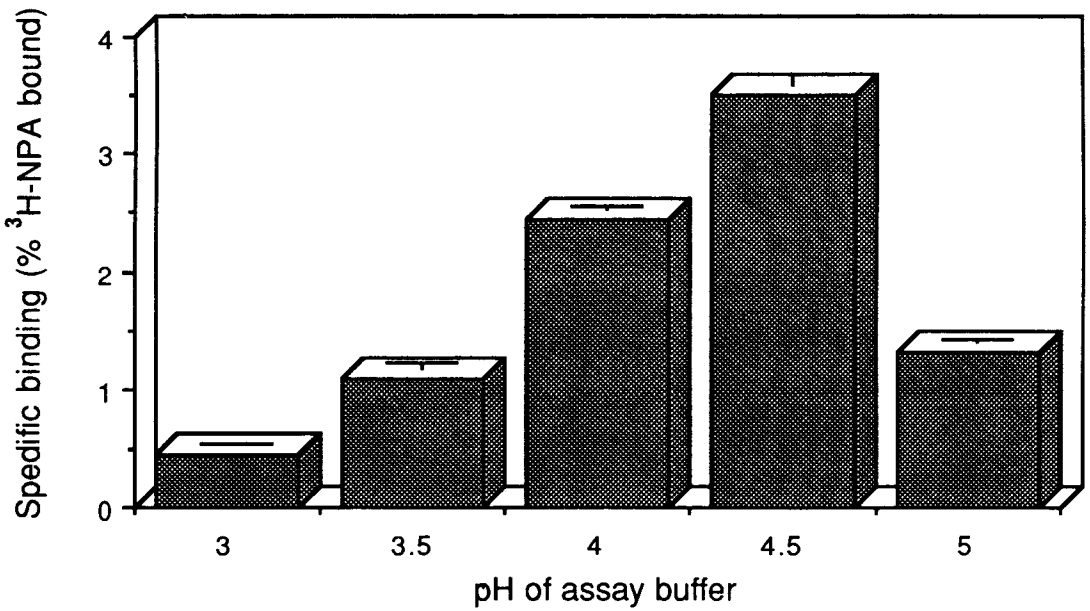


Fig. 3-2 Effect of pH of assay buffer on specific NPA binding

3.3.2. Effect of temperature on specific binding of ^{3}H-NPA

NPA binding assay was carried out at 4°C, 15°C and 21°C. Specific binding of NPA is sensitive to temperature, as illustrated in Fig. 3-3. The specific ^{3}H-NPA binding increased as the temperature was lowered. Both total binding and nonspecific binding were affected by the temperature treatments as shown in Table 3.1. These studies indicated that 4°C is the optimal temperature for specific ^{3}H-NPA binding.

Table 3.1 Effect of temperature on NPA binding*

Binding of NPA (dpm)	Temperature (°C) 4	15	21
Total binding	9040±106	8774±201	8449±86
Nonspecific binding	8291±71	8350±56	8133±138
Specific binding	749±19	424±27	316±30

*The error bars show standard deviation of the mean of four replicates.

3.3.3 Determination of binding kinetics

3.3.3.1 Affinity constant

The affinity constant for NPA binding was determined by Scatchard analysis (Scatchard, 1949). Fig. 3-4 shows a typical Scatchard plot which indicates a Kd of approximately 1.7 x 10^{-7} mol dm^{-3}. The steep part of the plot (left hand side) represents high affinity with

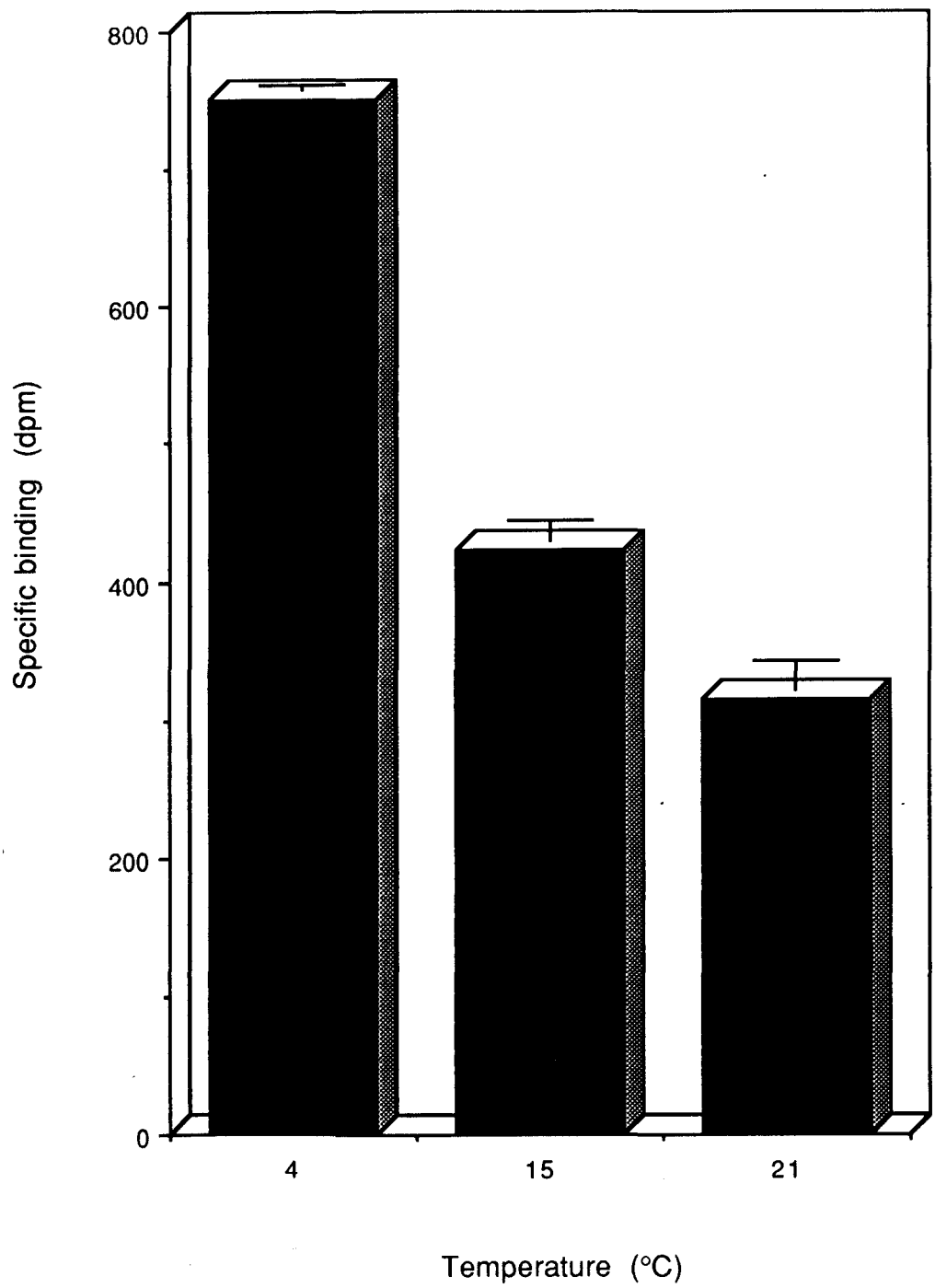


Fig. 3-3 Effect of temperature on specific NPA-binding

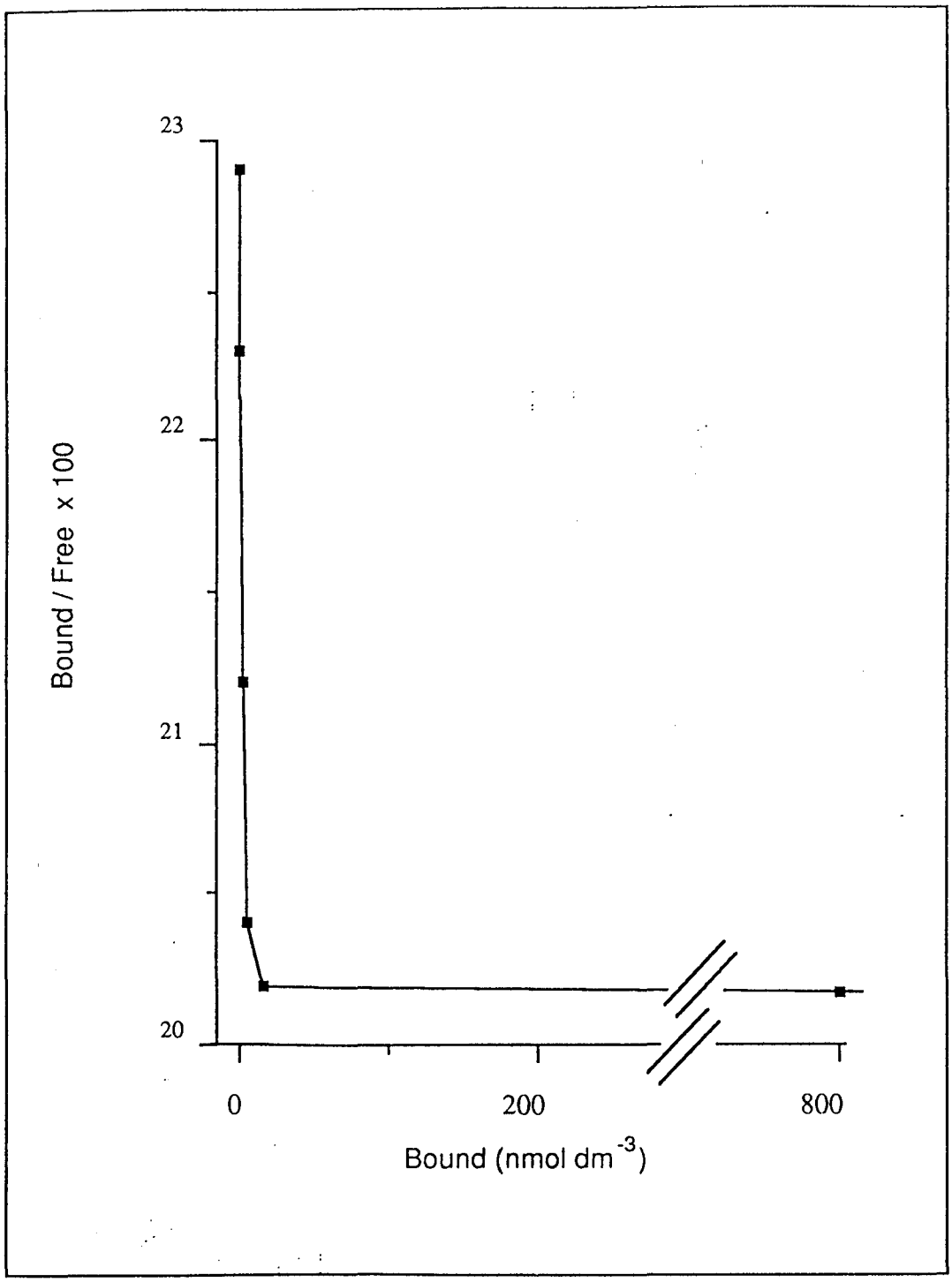


Fig. 3-4 Scatchard plot of NPA binding

low capacity binding, whereas the horizontal part indicates nonspecific binding with low affinity. The concentration of the binding sites was 0.22±0.007 (n=3) pmol mg^{-1} membrane protein. The calculation was made using the revised SCAPRE program (distributed by Institute of Nuclear Medicine, The Middlesex Hospital Medical School, London, U.K.).

3.3.3.2 Reversibility of NPA binding

With the filtration method, the reversibility of the NPA binding could be demonstrated by the time course of association and dissociation of ^{3}H-NPA. When 4 x 10^{-10} mol dm^{-3} ^{3}H-NPA was added to the membrane preparation, NPA and its binding sites began to associate immediately. As shown in Fig. 3-5, equilibrium was reached after about 55 min. As a result of competition after the addition of 10^{-4} mol dm^{-3} (final concentration) unlabelled NPA, ^{3}H-NPA dissociated from the binding sites within a few seconds. It seems that the amount of ^{3}H-NPA retained on the filter decreased upon the addition of higher concentrations of unlabelled NPA until almost no radioactive material above filter background was retained.

3.3.3.3 Competition experiments

Examples of displacement curves are given in Fig. 3-6. Auxins (2,4-D, α-NAA, ß-NAA and IAA) were all able to compete with ^{3}H-NPA for the NPA binding site. While the affinity constant Kd for others were around 10^{-6}-10^{-5} mol dm^{-3}, IAA was shown to have

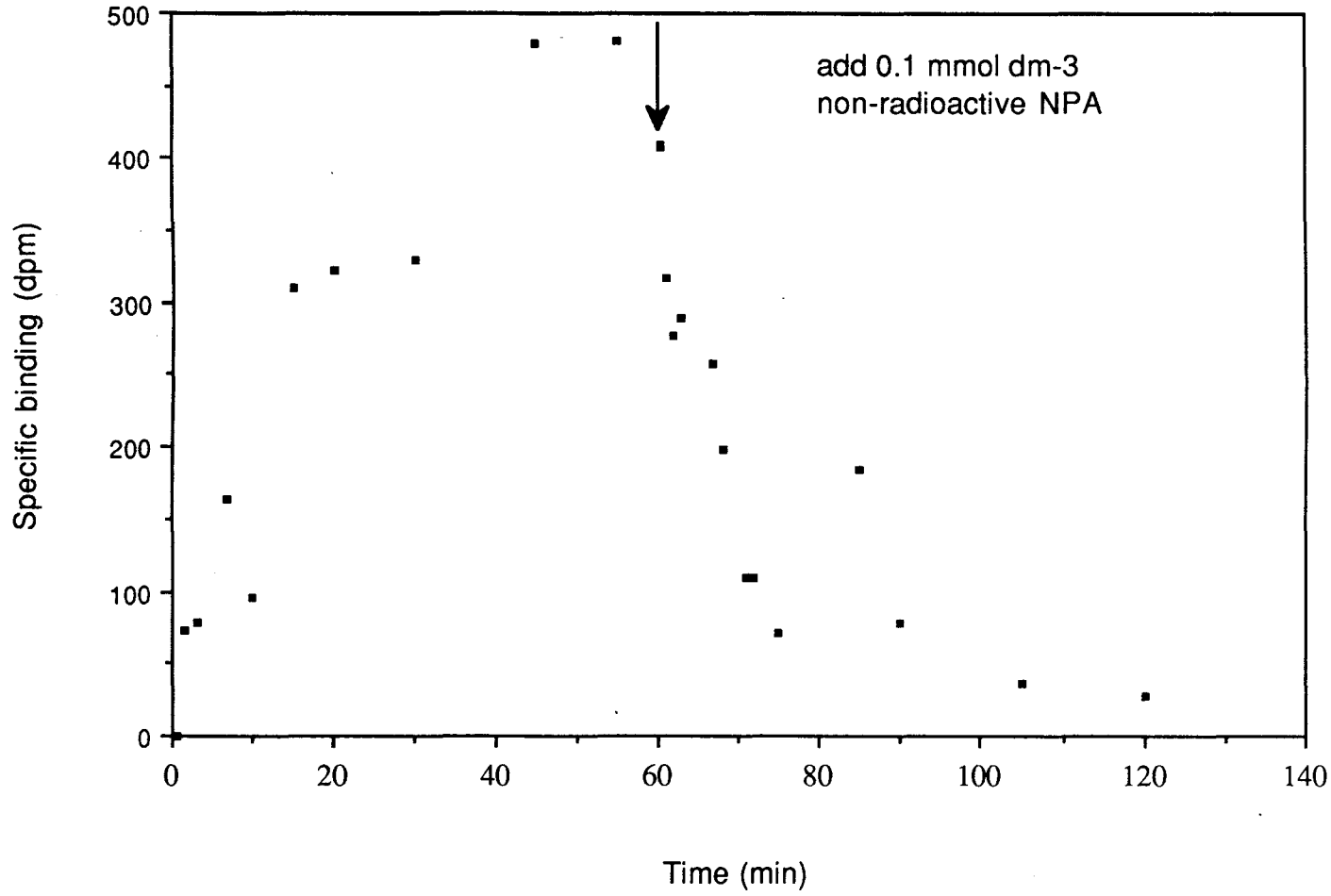


Fig. 3-5 Association and dissociation of NPA and its binding site

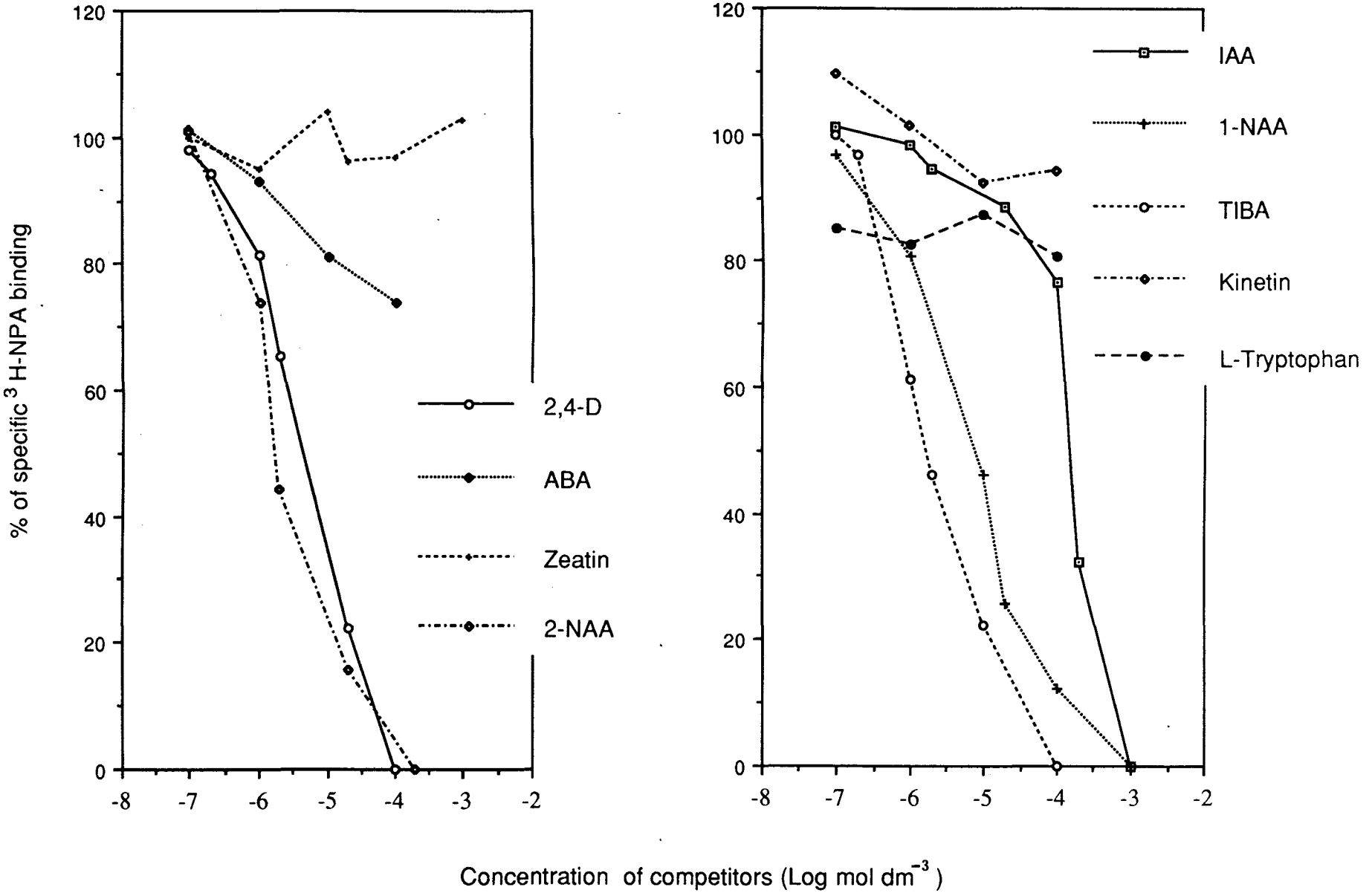


Fig. 3-6 Competition for the NPA binding site

only weak interaction with the NPA binding site, with a Kd above 5×10^{-4} mol dm^{-3}. Tryptophan, kinetin, zeatin and abscisic acid had no significant competition, even at 10^{-3} mol dm^{-3}. TIBA was found to be effective in displacing ^{3}H-NPA with a Kd of 10^{-6}-10^{-5} mol dm^{-3}. Since the concentration of competitor which gives 50% displacement concentration is a measure of the Kd value, these data also indicated the affinity of the NPA binding site for different compounds.

3.3.4 Activation of NPA binding with Mg^{2+} ATP

Cells were harvested 5 to 10 days after subculture. The membrane pellets were divided into two equal portions. Mg^{2+} ATP was added in one portion, giving a final concentration of 2 mmol dm^{-3} while the other was run as a control. In all four different experiments, higher specific NPA binding was found in the assays with Mg^{2+} ATP (Fig. 3-7). When expressed as a percentage of the specific binding, the extent of the increase in specific binding resulting from addition of Mg^{2+} ATP to the binding assays was always found to be greater than 30%.

3.3.5 Inactivation of NPA binding with acid phosphatase

Both NPA binding and acid phosphatase activity are likely to be influenced by temperature. Binding assays with the addition of acid phosphatase were performed at three temperatures, i.e. 4°C, which was shown to be optimal for NPA binding (Fig. 3-3), 37°C, at which acid

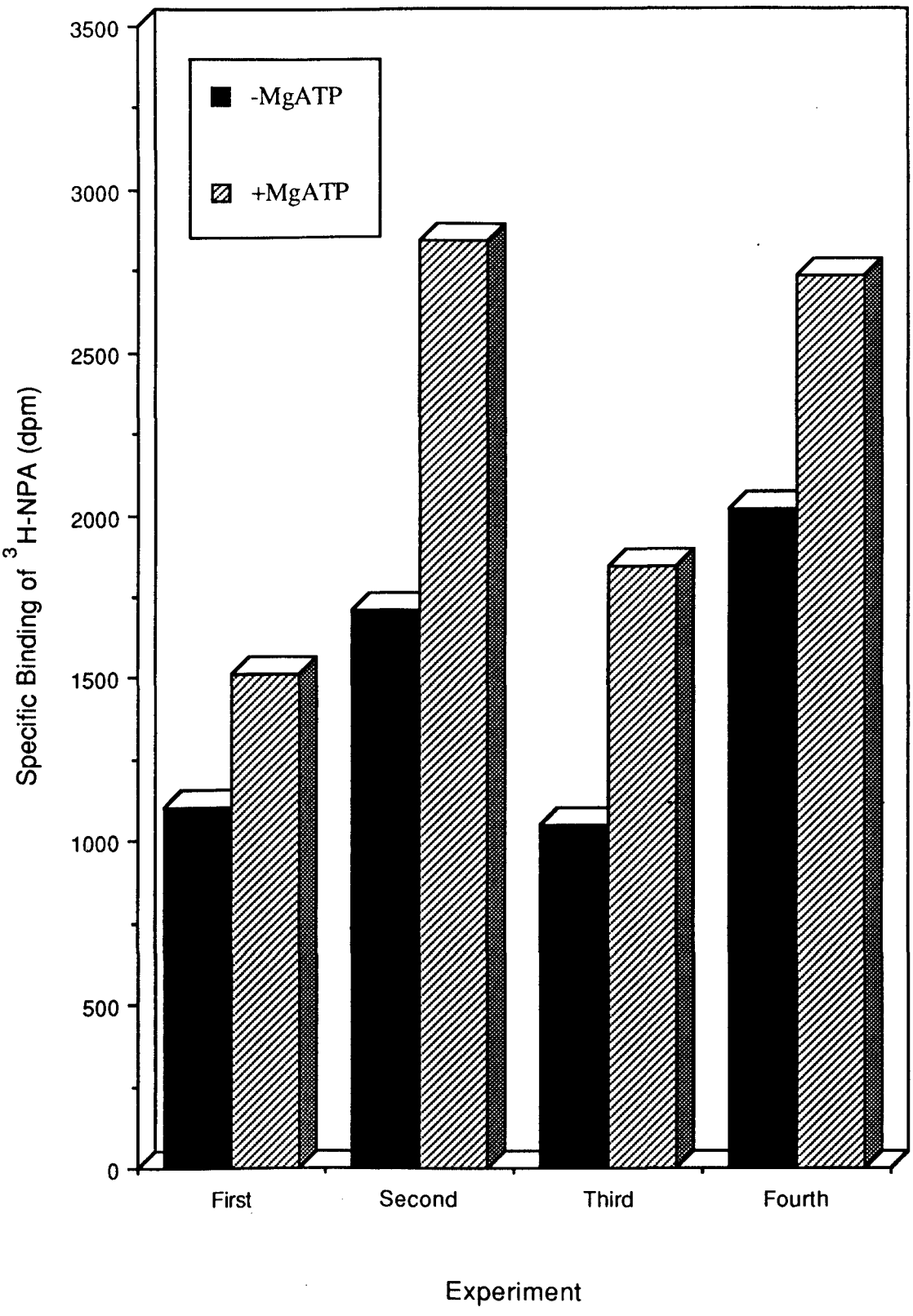


Fig. 3-7 Effect of Mg^{2+}ATP on specific NPA binding

phosphatase has maximal activity, and 20°C as a compromise condition. Acid phosphatase did not affect NPA binding at 4°C, whilst at 37°C binding in neither the control nor enzyme-treated samples could be detected. However, acid phosphatase had a clear effect at 20°C, even though the specific binding of the control was shown to be less than that at 4°C (Fig. 3-8). At this temperature, specific binding, under the effect of acid phosphatase treatment, decreased to 67% of that of the control. Further experiments on the effect of acid phosphatase were performed at 20°C. The effect of this enzyme on NPA binding was also shown to be concentration-dependent, with increase in enzyme concentration resulting in decreased specific NPA binding. Extended treatment also led to greater reduction of the specific binding. The effects of acid phosphatase concentration and its treatment duration are shown in Fig. 3-9. Boiled acid phosphatase (20 min) had no effect on specific NPA binding.

3.3.6 Culture stage dependence of Mg^{2+} ATP and acid phosphatase effects on NPA binding

The effects of Mg^{2+} ATP and acid phosphatase on NPA binding strongly imply that a phosphorylation/dephosphorylation mechanism operates in the regulation of the activity of NPA binding proteins. The level of NPA binding proteins was not always constant, as indicated in our previous studies in *Acer pseudoplatanus* L. cells (Elliott *et al.*, 1988b). One of the regulatory mechanisms could well be the extent of phosphorylation of this protein. Hence, the response of NPA binding to Mg^{2+} ATP and acid phosphatase treatment

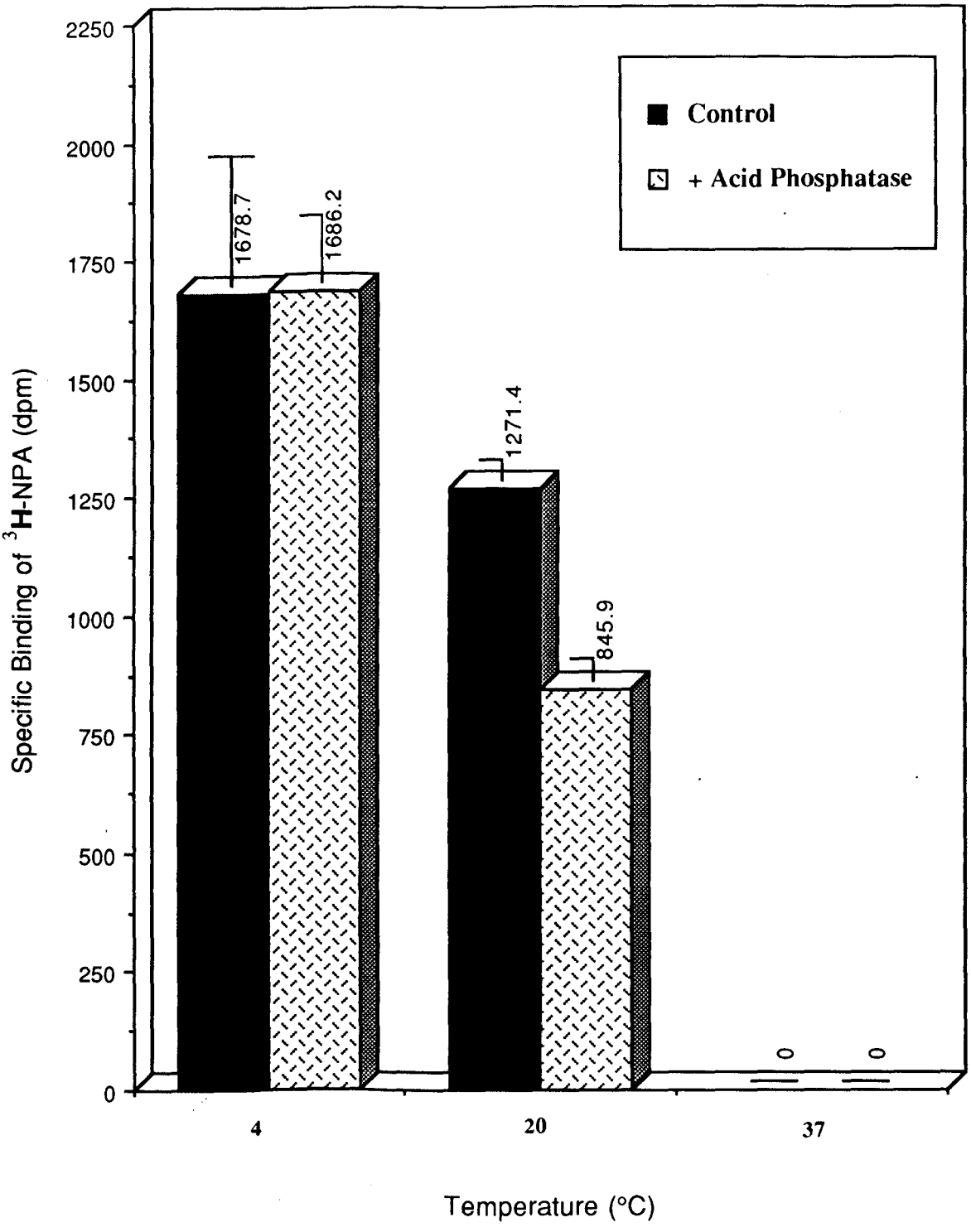


Fig. 3-8 Effect of acid phosphatase on NPA binding at different temperatures

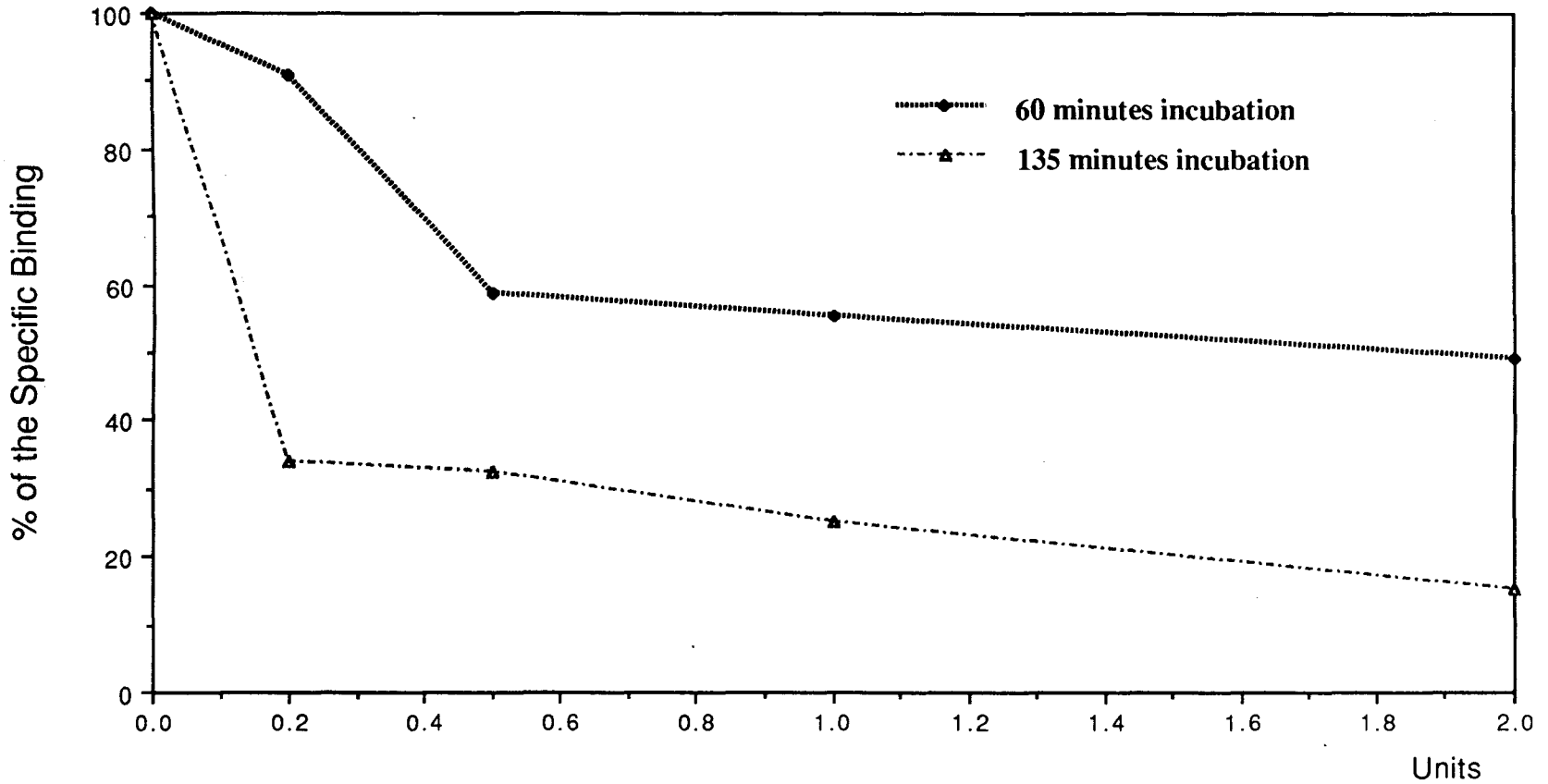


Fig. 3-9 Effect of acid phosphatase concentration and time on specific binding of NPA

was investigated in the growth cycle of the sugar beet cell suspension culture. Three representative stages were studied. These include day 1 (within the lag phase), day 8 (within the linear phase) and day 14 (within the stationary phase). While the effect could be reproducibly observed when using cells from day 8, there seemed to be no response to the same treatment when using cells from day 1 or day 14 (Fig. 3-10).

3.3.7 Endogenous phosphatase activity

Endogenous phosphatase may represent an internal mechanism which could dephosphorylate NPA binding proteins. After isolation of the membrane preparation, both the membrane pellet and the supernatant were collected. Phosphatase activity could not be detected in the membrane fractions, but high levels of the activity were reproducibly detected in the supernatant, using pNPP as its substrate at pH 8.0. The activity was shown to be 1.63 units per cm^3 of the supernatant. Considering the volume ratio of plant material and isolation buffer (1:1), the activity in the cytosol of intact cells can be expected to be even higher and exceed the concentrations of acid phosphatase that were used in the *in vitro* NPA binding assay. These observations highlight the possibility that the activity of the NPA binding protein could be regulated *in vivo* by dephosphorylation.

3.4 Discussion

3.4.1 Optimization of conditions for NPA binding

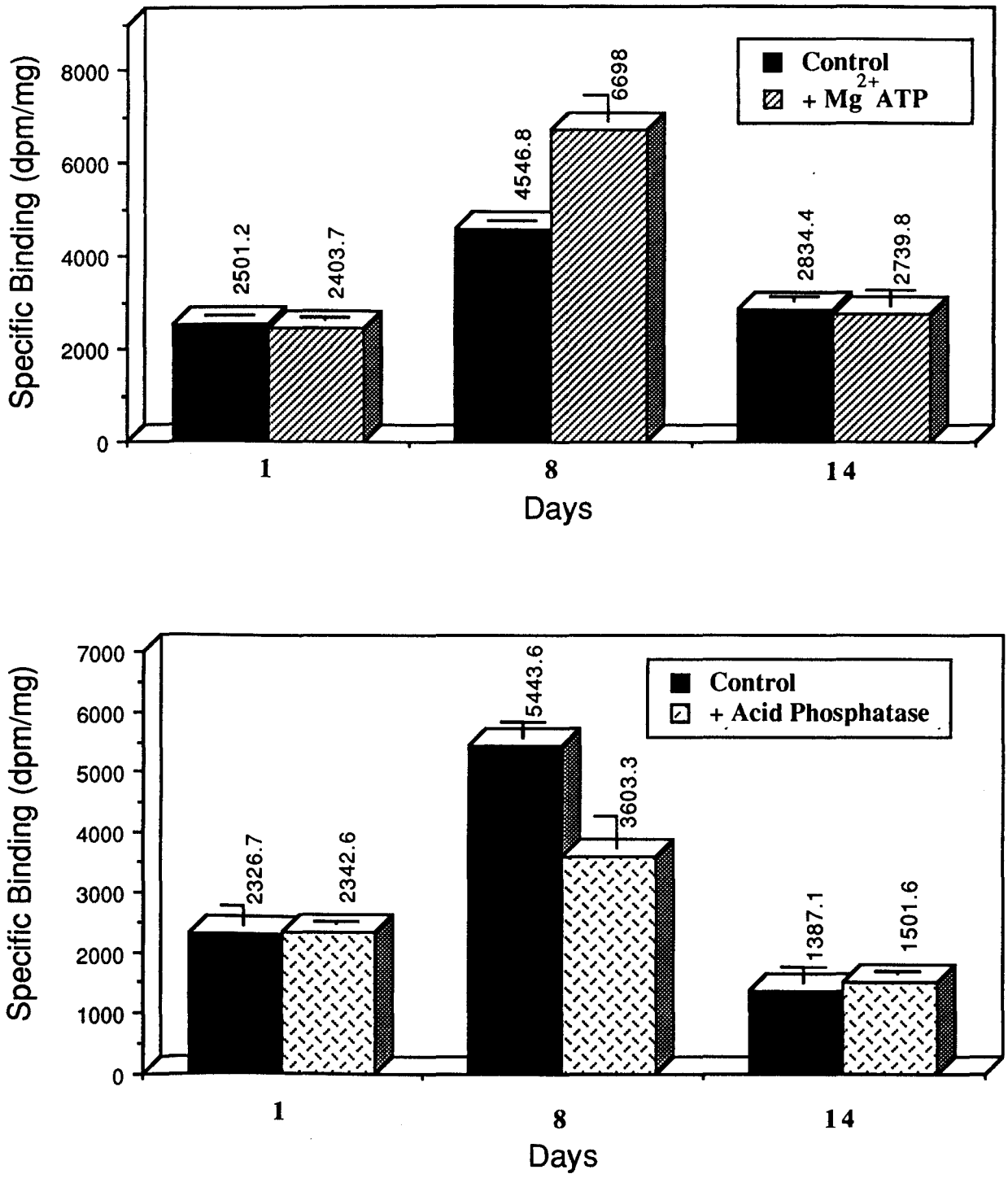


Fig. 3-10 Influence of time following cell transfer on effect of Mg ATP and acid phosphatase on specific NPA binding

The pH optima for the extraction and detection of NPA binding proteins in sugar beet seedling leaf cell suspension cultures were examined. At the membrane extraction and binding assay stages, the optimal pHs were determined to be 8.0 and 4.5 respectively. The optimal pH of the extraction buffer was similar to that observed for many of the membrane-bound ABPs studied in different plant species (Libbenga *et al.*, 1986). The optimal pH of the assay buffer was quite similar to that of the NPA binding proteins present in suspension cultured *Nicotiana* cells (Maan *et al.*, 1985), *Acer pseudoplatanus* L. cells (Elliott *et al.*, 1988b) and particulate cell fractions of corn coleoptiles (Thomson *et al.*, 1973).

Compared with the pHs of all three major compartments in the system (cytoplasm, vacuole and culture medium, e.g. Elliott *et al*, 1988b), this optimum pH for NPA binding could be considered much lower than that expected for *in vivo* binding. The pH optima for auxin binding to fruit tissues and vegetative tissues ranged from 3.5 in bean, strawberry and tomato fruit crude membrane fraction (Mudge, 1980; Narayanan *et al.*, 1981), 3.75 in cucumber fruit (Mudge, 1980), 5.0 in zucchini (Jacobs, 1978) and tobacco pith callus (Vreugdenhil *et al.*, 1979), 5.5 in corn (Batt and Venis, 1976; Ray *et al.*, 1977), to 8.0 in mung bean (Kasamo and Yamaki, 1976; Wardrop and Polya, 1980). Membrane-bound NPA binding activity in maize coleoptiles had a pH optimum of 5.0 (Thomson *et al.*, 1973; Sussman and Gardner, 1980), while solubilized NPA binding activity in the same material had an optimum near pH 4.0 (Sussman and Gardner, 1980). Meanwhile, Sussman and Gardner (1980) found a striking result that this binding site in membrane-bound and

soluble states had a similar pH dependence above pH 5.0, but the soluble protein was active at lower pH values, whereas the native protein was not.

The temperature optimum for membrane-bound NPA binding proteins in sugar beet seedling leaf cell suspension culture was shown to be at low temperature. Compared to the NPA binding activity at 15°C and 21°C, it was found that a much higher specific binding was obtained at 4°C. Most of the membrane-bound ABPs reported in the literature were performed at or about 0°C (Ray *et al.*, 1977a). The main reason for this non-physiological temperature is that under *in vitro* conditions the membrane-bound auxin binding proteins of these plants are thermolabile, as has clearly been shown in corn coleoptile tissue (Ray *et al.*, 1977a; Löbler and Klämbt, 1985). Comparison of the properties of membrane-bound and solubilized NPA binding proteins revealed that the NPA binding activity in the soluble state was more heat-labile than that in membrane-bound state (Sussman and Gardner, 1980). Thus, whilst this data indicates the optimum temperature for *in vitro* binding, this may be a consequence of extraction and have limited significance for the *in vivo* circumstance.

3.4.2 Kinetics of specific NPA binding

Scatchard analysis of membrane-bound NPA binding data indicated a high affinity binding protein with a dissociation constant (Kd) of 1.71×10^{-7} mol dm^{-3}. This value was similar to those reported in maize coleoptiles (Thomson, 1972; Normand *et al.*, 1975; Sussman

and Gardner, 1980), but was higher (lower affinity) than those found in suspension cultured *Nicotiana* cells (Maan *et al.*, 1985) and *Acer pseudoplatanus* L. cells (Elliott *et al.*, 1988b).

In competition experiments designed to determine the specificity of the NPA binding protein, α-NAA, ß-NAA and 2,4-D were found to be able to compete with NPA (Kd from 10^{-6} to 10^{-5} mol dm^{-3}), whilst IAA was shown to have only weak interaction with the NPA binding protein (Kd>10^{-4} mol dm^{-3}). Tryptophan, kinetin, zeatin and abscisic acid were virtually not competitive. Similar results demonstrating weak competition by IAA for NPA binding to membranes have been reported in maize coleoptile (Sussman and Gardner, 1980), suspension cultured *Nicotiana* cells (Maan *et al.*, 1985) and *Acer pseudoplatanus* L. cells (Elliott *et al.*, 1990). There are several possible explanations for the low affinity of IAA to NPA binding proteins.

Firstly, if we consider NPA binding proteins as IAA efflux carriers, we could expect that these binding sites had higher affinity for IAA on the inside of the plasma membrane than that on the outside so that IAA would be released on the outside of the cells into the cell-wall free surroundings. This high affinity for IAA on the inside of the plasma membrane might not be detected at low assay pH shown to be optimal for *in vitro* binding assay of NPA, whereas at cytoplasmic pH (approximately 7.4) which might favour IAA binding of these carriers NPA binding would not be detected. Results with the solubilized NPA binding site from corn coleoptile showed that the affinity for IAA was at least 100

times higher in the solubilized sites than in the non-solubilized ones. This result may show the importance of the subcellular environment, such as membrane structure, on the observed binding affinity. The solubilization procedure could have changed the fine structure (conformation) of the carrier and eased the access for IAA to bind to them.

Secondly, two distinct sets of NPA binding proteins have been identified, of which only those of lower affinity appear to be directly related to the transport process (Michalke *et al.*, 1992). In this context, the affinity of IAA to the transport-related set of NPA binding protein can be expected to be lower than would be expected if the first explanation was correct. Jacobs and Rubery (1988) pointed out that certain flavonoids (e.g. quercetin) could specifically compete with ^{3}H-NPA for the binding protein with Kd of 5 x 10^{-5} mol dm^{-3} in zucchini hypocotyls. Such active flavonoids are widely distributed in the plant kingdom and exert their effects at micromolar concentrations approximating likely endogenous levels. While their binding affinities are orders of magnitude lower than that of the NPA; this does not necessarily debar their physiological significance, especially in view of the observation from Michalke *et al.* (1992). It is of interest to consider the possibility that the genuin affinity of IAA to the efflux carriers has evolved to be low in order that intracellular IAA level can be economically maintained, precisely adjusted and efficiently utilized for cell growth. This affinity level could well be around 10^{-5} mol dm^{-3} of Kd as found in the solubilized NPA binding proteins by Sussman and Gardner (1980).

Thirdly, Depta and Rubery (1984) and Depta (1987) proposed a "three site" model for

auxin efflux carrier: this carrier complex consists of an auxin translocation site plus two distinct regulatory sites with differential affinities for NPA and 2,3,5-TIBA. It is suggested that the NPA binding site is distinct from the site of auxin translocation, though the two must be in close association, e.g. as subunits of a complex. *In vitro* competition experiments described by previous workers (e.g. Maan *et al.*, 1985; Elliott *et al.*, 1990; Sussman and Gardner, 1980) can be expected to fail to detect a high level of displacement of ^{3}H-NPA binding by IAA, because the NPA binding site in the efflux complex was suggested not to bind auxin as proposed in the model. Any *in vitro* study, through binding assays, on the ralation of the "three sites", is technically difficult and complicated, and the model was based on *in vivo* transport experiments (Depta and Rubery, 1984; Depta, 1987).

3.4.3 The role of phosphorylation in control of NPA binding protein activity

Our previous work with *Acer pseudoplatanus* L. cell suspension cultures suggested that NPA binding could be involved in the regulation of intracellular IAA levels and cell division (Elliott *et al.*, 1988b). These findings may indicate that the NPA binding protein functions as a regulatory element in the control of auxin transport. As the initial point of interaction of growth regulators, the regulation of the receptors themselves are of particular importance. However, the regulation of this key point in the signal transduction chain has not received much attention in the study of plant hormone receptors. In our present work, we initially examined the possible role of phosphorylation/dephosphorylation in the

regulation of the activity of NPA binding proteins, as this regulatory mechanism has been proposed to be universal for membrane receptors in animal systems (Huganir and Greengard, 1987).

This regulatory mechanism was investigated by separate additions of Mg^{2+} ATP, and acid phosphatase, to the binding assay. These treatments were found to considerably affect NPA binding. High level of phosphatase activity was detected in the intact cells.

In plant systems, preliminary work reported at the 1984 FEBS Congress (van der Linde *et al.*, 1985) suggested that inactivation of soluble auxin binding proteins by phosphatase may largely contribute to the lack of reproducibility in detecting this protein. Phosphatase was present in concentrated cytosol of tobacco tissues as indicated by its ability to hydrolyse pNPP. Excess substrate for phosphatases was tried in an attempt to protect this protein against attack by phosphatases. The phosphatase-inactivated binding protein should also be able to be reactivated by protein kinases. As expected, both pNPP and Mg^{2+} ATP increased the level of the binding protein.

In a study of membrane-bound fusicoccin binding protein, the instability of this binding protein was suggested to be caused by attack by endogenous phosphatase and α-mannosidase (Aducci *et al.*, 1984). The high level of phosphatase activity in the cytosol of our sugar beet cells indeed supports our proposal that the activity of NPA binding protein could be regulated by the endogenous phosphatase in the intact cells. The effect

of Mg^{2+} ATP on the reactivation or recovery of this binding strongly implies that phosphorylation (presumably through protein kinase action) as well as dephosphorylation may co-operate in the regulation of NPA binding activity.

The effects of Mg^{2+} ATP and acid phosphatase on NPA binding was examined at three representative periods of the batch culture cycle. It is notable that neither Mg^{2+} ATP nor acid phosphatase caused any change of specific NPA binding when using cells from lag phase or stationary phase of the batch culture, whilst they substantially altered the activity when using cells from the linear phase. The significance of these observations have to be elucidated.

It is generally agreed that NPA binding proteins are preferentially located at the basal ends of auxin transporting cells (Rubery, 1987). In animal systems, at least two major effects of receptor phosphorylation have been identified --- regulation of receptor function and regulation of receptor distribution. The latter was found with the ß-adrenergic receptor, and receptors for insulin, epidermal growth factor, insulin-like growth factor II and transferrin ((Sibley *et al.*, 1987). By analogy with animal systems (Klausner *et al.*, 1984; May *et al.*, 1984, 1985) it is possible that phosphorylation/ dephosphorylation has a regulatory function in polar auxin transport. The data presented here is consistent with regulation by phosphorylation/dephosphorylation of the function of the NPA binding proteins.

Chapter 4 Molecular Characterization of an Auxin Binding Protein

4.1 Introduction

The first publications on binding of auxin by specific binding proteins were those by Hertel and co-workers (Lembi *et al.*, 1971; Hertel *et al.*, 1972). These reports stimulated research on plant hormone binding proteins. The maize coleoptile system has proved to be convenient and to yield reproducible results. The auxin binding protein in maize coleoptile membranes has been extensively investigated in several laboratories and appears to fulfil many of the criteria expected of a genuine receptor (see Napier and Venis, 1991).

A high degree of purification was reported for ABP by Löbler and Klämbt (1985a) and since then several groups have established purification protocols (Shimomura *et al.*, 1986; Napier *et al.*, 1988), raised antibodies (Löbler and Klämbt, 1985b; Napier *et al.*, 1988), and made progress towards understanding the function of this protein.

Oligonucleotides synthesised on the basis of the *N*-terminal amino acid sequence of purified ABP were used to screen a maize cDNA library, yielding a partial-length clone (Inohara *et al.*, 1989). By rescreening with this clone, several further clones were isolated, including one that was full-length. The sequence indicates a 38 amino acid signal peptide, followed by the 163 residues of the mature protein. ABP shows no homology with any other known sequence and the auxin-binding site has yet to be identified. Other laboratories have

obtained very similar sequence data (Hesse *et al.*, 1989; Tillmann *et al.*, 1989; Yu and Lazarus, 1991).

All the work described above was on maize. In our present work, sugar beet (*Beta vulgaris* L.) seedlings were chosen as our experimental material because of the agricultural and commercial importance of this species and the background knowledge accumulated in this laboratory (Elliott *et al.*, 1988a). Purification was to be based on that of Shimomura *et al.* (1986), which includes successive chromatographies on DEAE-Sephacel, α-NAA-linked AH-Sepharose 4B, and Sephadex G-100 columns. Also, a maize ABP cDNA (kindly provided by Dr. C.M. Lazarus of Bristol University) was available to be used as a probe to screen sugar beet genomic DNA for homologous sequences by Southern hybridization. It was hoped that the use of this probe would help to isolate gene(s) coding ABP in sugar beet by enabling sugar beet cDNA or genomic DNA libraries, which are available within the laboratory, to be screened. Both ABP purification and ABP gene isolation will further our understanding of its function and eventually contribute to our effort in plant growth regulation through hormone receptor manipulation.

4.2 Materials and methods

4.2.1 Plant materials

Seedlings of maize (*Zea mays* L. cv. Marada) and sugar beet (*Beta vulgaris* L. cv. Regina)

were grown in 25-cm pots in the greenhouse under supplemental lighting to provide a 16-hr photoperiod. Maize leaves (3-4 weeks old) were harvested and frozen with liquid nitrogen for DNA extraction. Sugar beet seedlings (3 weeks old) were harvested for membrane isolation or frozen with liquid nitrogen for DNA extraction.

4.2.2 Preparation of solubilized microsomal extracts

Sugar beet seedlings were chopped briefly and homogenised in an ultra Turrax homogenizer for about 20 sec in short bursts. The homogenization buffer contained 50 mmol dm^{-3} Tris/HCl, 1.0 mmol dm^{-3} Na_2EDTA, 0.10 mmol dm^{-3} $MgCl_2$, 14 mmol dm^{-3} ß-mercaptoethanol and 0.25 mol dm^{-3} sucrose (pH 8.0). The fluid was pressed through nylon cloth and the microsomes were prepared by sequential sedimentation at 4,000*g* (10 min) and 50,000*g* (45 min) as described in section 3.2.2. The microsomal pellet was then resuspended in 10 mmol dm^{-3} sodium citrate/citric acid, 0.5 mmol dm^{-3} $MgCl_2$ and 0.25 mol dm^{-3} sucrose (pH 5.5). The whole isolation procedure was carried out at 4°C or on ice.

To obtain a detergent-solubilized preparation, the resuspended microsomal fraction was mixed with 1% (w/v) TritonX-100 and incubated for 30 min at 4°C. After centrifugation at 110,000*g* for 1 hr, the supernatant (representing the solubilized extract) was removed carefully to avoid disturbing the pellet.

Acetone was used to solubilize the auxin-binding protein according to the procedure of

Venis (1977). The microsomal suspension was rapidly added to a centrifuge bottle containing 20 volumes of rapidly stirred acetone at -20°C. The precipitate was then pelleted at 4,000*g* for 10 min. The supernatant was decanted, and the drained pellet was dried under a stream of nitrogen at 4°C. The dried acetone-washed membrane was homogenized with the assay buffer (10 mmol dm^{-3} sodium citrate/citric acid, 5 mmol dm^{-3} $MgCl_2$, 0.2 mmol dm^{-3} phenylmethylsulfonyl fluoride, pH 5.7) and the volume was kept at a ratio of 1 cm^3/10 g of fresh seedlings. After centrifugation at 110,000*g* for 1 hr, the supernatant (representing solubilized extract) was carefully removed.

4.2.3 Detection of auxin binding protein in solubilized extracts

The supernatant was incubated with 10^{-9} mol dm^{-3} ^{3}H-IAA (specific activity 999 GBq $mmol^{-1}$, Amersham, U.K.) ± 10^{-4} mol dm^{-3} unlabelled IAA, in a final volume of 1 cm^3, giving ca. 5 g f.wt. of tissue per sample. The binding mixture was incubated at 4°C for 60 min and then specific binding was determined with the following techniques.

Gel filtration: A gel filtration technique for binding assays has been reported (Hummel and Dryer, 1962; Goldstein and Blecher, 1976; Cross *et al.*, 1978; Sussman and Gardner, 1980). The protein-bound hormone will appear in the excluded volume, separated from the later-eluting peak of free material.

Prepacked, disposable columns (PD-10; bed volume of 9 cm^3) of Sephadex G-25M

(Pharmacia) were used in the binding assay. Each column was equilibrated with at least 30 cm^3 resuspension buffer just before applying the solubilized binding mixtures (0.5 cm^3) onto the column. As soon as the top of the column was dry, the entire void volume was eluted with an additional 6.5 cm^3 resuspension buffer. A total volume of 7.0 cm^3 was collected, ready for counting of radioactivity. Initially, control samples containing blue dextran 2000 (mol wt of 2 x 10^6) and ^{3}H-IAA were applied to columns for calibration of their void volumes and to ensure that free IAA was adequately resolved from protein present in the void peak. All experiments were performed at 4°C.

Centrifree™ MPS-1 Micropartition: The disposable Centrifree™ Micropartition System (Amicon Ltd., U.K.) has been specifically designed for separation of free from protein-bound microsolute. It provides a method for rapid partition of free ligand from small volumes (0.15-1.0 cm^3) of biological samples while maintaining physiological equilibrium conditions.

This system has been successfully used for the determination of NPA binding proteins (Jacobs and Gilbert, 1983) and auxin binding proteins (Venis, 1987). An aliquot (0.5 cm^3) of incubation mixture was applied to the sample reservoir by holding the pipette at a slight angle to avoid touching the membrane with the pipette tip. The samples were centrifuged through the YMT membrane by mounting the MPS-1 assemblies in a refrigerated centrifuge (4°C) (at 2,000*g* for 30 min). The filtrate cups were carefully removed from the device and 250 mm^3 of the ultrafiltrate from each sample was taken for radioactivity counting.

4.2.4 Purification of auxin binding protein

Auxin binding protein was solubilized from the acetone-washed membranes and purified by chromatographies on DEAE-Sephacel, α-NAA-linked AH-Sepharose 4B and Sephadex G-200 columns (Pharmacia Ltd.).

Ion exchange chromatography: The separation by ion exchange chromatography was obtained by reversible adsorption. Most ion exchange experiments are performed in two main stages. The first stage is sample application and adsorption. Unbound substances can be washed out from the exchanger bed using a column volume of starting buffer. In the second stage, substances are eluted from the column, separated from each other. Separation is obtained providing different substances have different affinities for the ion exchanger due to differences in their charge. Ion exchange chromatography is capable of separating molecules with very small differences in charge, and is therefore a very powerful separation technique.

Solubilized extracts from acetone-washed membrane were applied to a DEAE-Sephacel column (1.5 x 10 cm) equilibrated with TM buffer (10 mmol dm^{-3} Tris/HCl, 5 mmol dm^{-3} $MgCl_2$, pH 7.2). After washing the column with 100 cm^3 TM buffer, the auxin-binding activity was eluted with 40 cm^3 TM buffer containing 0.3 mol dm^{-3} NaCl. This eluate was concentrated by ammonium sulphate (75% saturation) and auxin-binding activity was determined by Centrifree ™ MPS-1 micropartition. Non-saturable binding (determined in

the presence of 10^{-4} mol dm^{-3} unlabelled NAA) was subtracted from total ^{14}C-NAA binding (5×10^{-9} mol dm^{-3} ^{14}C-NAA, specific activity 92.5 MBq $mmol^{-1}$, Amersham, U.K.) to give saturable binding.

Affinity chromatography: The basic requirement of an affinity chromatography is the design of a specific adsorbent consisting of a solid support to which is covalently attached a ligand (e.g. hormone or analogue or antagonist) with high affinity for the receptor to be isolated. Ideally, if a crude mixture is passed through the adsorbent, only the receptor will be bound, contaminating proteins will pass straight through, and the pure receptor can then be recovered by changing the elution conditions. NAA-Sepharose 4B matrix has been successfully used in purification of membrane-bound ABP (Shimomura *et al.*, 1986) and and 2,4-D-Sepharose 4B matrix in the purification of sABP (Bailey *et al.*, 1985; Barker and Bailey, 1988). AH-Sepharose 4B (Pharmacia Ltd.) has free primary amino groups at the end of 6-carbon spacer arms for coupling ligands containing free carboxyl groups. The coupling procedure of NAA-linked AH-Sepharose 4B is shown in Table 4.1.

The solubilized extract was applied to the NAA-linked AH-Sepharose 4B column (1.5 x 10 cm) equilibrated with TM buffer. The column was eluted with 80 cm^3 TM buffer containing 0.5 mol dm^{-3} NaCl and then with 30 cm^3 TM buffer containing 0.5 mol dm^{-3} NaCl plus 10^{-3} mol dm^{-3} α-NAA. Since NAA at a high concentration interfered with both the auxin binding assay and protein assay, the fraction eluted with NAA was sufficiently diluted with TM buffer and then concentrated by ammonium sulphate (75% saturation).

Table 4.1 Preparation of NAA-linked AH-Sepharose 4B for affinity chromatography

Step	Note
weight out required amount of AH-Sepharose 4B	20 cm^3 swollen AH-Sepharose 4B corresponds to 7 g of dry weight material.
wash and reswell on a sintered glass filter	Use 1 litre 0.5 mol dm^{-3} NaCl for washing; stir gently then suck through the filter. The gel is washed again with distilled water adjusted to pH 4.5.
dissolve α-NAA to be coupled	200 mg α-NAA is dissolved in 20 cm^3 dioxane and water (H_2O pH adjusted to 4.5) mixture (1:1).
dissolve carbodiimide in water and adjust to pH 4.5	500 mg water soluble carbodiimide, i.e. *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is dissolved in 10 cm^3 water.
mix ligand solution and carbodiimide solution with gel suspension in an end-over-end mixer	α-NAA solution is added to the gel and the pH is adjusted to between 4.5 and 6.0. The EDC solution is then added dropwise as an aqueous solution. The suspension is gently mixed and the pH value should be adjusted during this time by addition of dilute NaOH solution. The reaction takes place overnight at room temperature.
to remove the excess uncoupled ligand that remains after coupling	The final product should be washed with a mixture of dioxane and water (1:1) followed by a wash with distilled water and then an alternative wash for four or five times with acetate buffer (0.1 mol dm^{-3}, pH 4.0) and $NaHCO_3$ buffer (0.1 mol dm^{-3}, pH 8.3), each containing 0.5 mol dm^{-3} NaCl.
The NAA linked AH-Sepharose 4B conjugate is finally equilibrated with TM buffer (pH 7.2) and ready for use or stored at 4-8°C.	

Centrifree MPS-1 micropartition was used for the binding assay.

The gel was regenerated as below: (1) wash with 10 column volumes of 0.1 mol dm^{-3} Tris/HCl buffer containing 0.5 mol dm^{-3} NaCl, adjusted to pH 8.5; (2) wash with 10 column volumes of 0.1 mol dm^{-3} sodium acetate buffer containing 0.5 mol dm^{-3} NaCl, adjusted to pH 4.5; (3) re-equilibrate with starting buffer.

Gel filtration: Gel filtration is often a very useful technique for fractionating proteins on the basis of size. It has been widely used in biochemistry research since it was introduced by Porath and Flodin (1959). The separation in gel filtration depends on the different abilities of the various sample molecules to enter pores which contain the stationary phase. Very large molecules which never enter the stationary phase, move through the chromatographic bed fastest. Smaller molecules, which can enter the gel pores, move more slowly through the column, since they spend a proportion of their time in the stationary phase. Molecules are, therefore, eluted in order of decreasing molecular size.

A column of Sephadex G-200 column (4.5 x 30 cm) was pre-equilibrated with TM buffer containing 0.1 mol dm^{-3} NaCl. The solubilized extract was chromatographed on the column by eluting with the same solution. Eighty fractions of 5 cm^3 were collected. Groups of 5 successive fractions were pooled and concentrated for auxin binding assay as described above.

4.2.5 Isolation of genomic DNA

4.2.5.1 Preparation of phenol and chloroform solutions

A. Phenol

1. The upper aqueous layer from a 1 dm^3 bottle of water-equilibrated phenol was removed, discarded and replaced by 1 mol dm^{-3} Tris-HCl, pH 8.0. The liquid phases were mixed by inverting the bottle several times.

2. After allowing the layers to separate, the upper aqueous phase was removed and discarded. It was replaced by 50 mmol dm^{-3} Tris-HCl, pH 8.0. The mixture was agitated and immediately 10 cm^3 samples were placed in 15 cm^3 centrifuge tubes and stored at -20°C.

3. Aliquots were let to thaw at 20°C prior to use. It was the dense lower organic layer which was used for treatment of DNA. The phenol remains stable at 4°C for about a month, but storage at room temperature or exposure to light enhances oxidation and the liquid becomes pink. Oxidation can be inhibited by addition of 8-hydroxyquinoline at 0.1% (w/v). This has the advantage of giving a bright yellow colour which identifies the organic layer during phenol extractions.

B. 24:1 (v/v) chloroform-isoamyl alcohol

96 cm^3 chloroform was mixed with 4 cm^3 isoamyl alcohol and stored in a capped bottle.

C. "Phenol-Chloroform"

A 10 cm^3 aliquot of phenol was thawed and 5 cm^3 of the lower phase was transferred to a universal bottle. This was mixed with 5 cm^3 of 24:1 (v/v) chloroform-isoamyl alcohol and 10 cm^3 TE buffer (10 mmol dm^{-3} Tris-HCl, 1 mmol dm^{-3} EDTA, pH 8.0). The solution was stored at 4°C. The lower organic layer was used.

4.2.5.2 Isolation of DNA by phenol-chloroform extraction

Genomic DNA was extracted from sugar beet and maize seedling leaves, based on a method described by Shure *et al.* (1983).

1. 10 g unexpanded leaves were placed into liquid nitrogen; 6 g alumina powder (Sigma type A-5) was added and the material was ground to a fine powder.

2. Acid-washed PVPP slurry (equivalent to 1 g PVPP dry wt.) and ca. 3 volume extraction buffer were added and the material was ground again. (extraction buffer: 5 mol dm^{-3} NaCl, 6.25 cm^3; 1 mol dm^{-3} Tris-HCl pH 8.0, 5.0 cm^3; 0.5 mol dm^{-3} EDTA pH 8.0, 4.0 cm^3; 20%

w/v Sarcosine, 5.0 cm^3; urea (do not autoclave), 42.0 g; add sarcosine and urea when ready to use; make up to 100 cm^3 with sterile distilled water).

3. The material was transferred to 50 cm^3 centrifuge tubes and an equal volume of phenol-chloroform was added.

4. The tubes were shaken well and left for 15 min.

5. They were centrifuged at 1000 *g* for 10 min at 4°C.

6. The aqueous phase (top) was transferred to new tubes.

7. 1/6 volume of 4.4 mol dm^{-3} NH_4Ac (pH 5.2) and equal (total) volume isopropanal (-20°C) were added and the tubes were inverted gently (DNA precipitation can be seen at this stage).

8. The DNA was spun down at 1000 *g* for 10 min at 4°C.

9. The resulting pellet was dried in a vacuum dessicator. The DNA was resuspended in 500 mm^3 TE buffer and transferred to an Eppendorf tube.

10. An equal volume of phenol-chloroform was added and mixed.

11. The DNA was spun down at 13,000 rpm for 10 min in a microfuge.

12. The aqueous phase (top) was removed to a fresh tube and steps 10-12 were repeated.

13. The wash with phenol-chloroform was changed to wash with chloroform only and steps 10-12 were repeated once.

14. The aqueous phase (top) was removed and the volume was estimated.

15. DNA was precipitated with 1/10 volume 3 mol dm^{-3} NaAc (pH 5.2) and 2.5 volume ice-cold absolute ethanol.

16. The solution was stored at -20°C overnight.

17. The DNA was spun down at 13,000 rpm in a microcentrifuge for 10 min. The precipitate was washed with ice-cold 70% ethanol.

18. The DNA was dried in vacuum dessicator, resuspended in 100 mm^3 sterile water and stored at -20°C.

4.2.6 Spectrophotometric determination of DNA

1. 10-100 mm^3 of the DNA sample was transferred to 900-990 mm^3 TE pH 7.5 in a 1 cm^3 quartz cuvette and the contents were mixed thoroughly. A spectrum from 250-320 nm was recorded. A smooth peak should be observed with an absorption maximum at around 260 nm and no noticeable shoulder at 280 nm. The 260:280 nm absorbance ratio should be more than 1.8. If the ratio is less than 1.8 then contamination with protein was suspected.

2. The quantity of DNA was calculated using the guide that 1 cm^3 of a solution with an A_{260} of 1.0 is equivalent to 50 μg of double-stranded or 35 μg of single-stranded DNA.

4.2.7 Assessment of the quality of DNA

The nucleic acid sample contains RNA contaminants. RNA can be removed by incubation with RNase A for 20 min at 37°C. (Commercial supplies of RNase A usually contain DNase activity. DNase-free stock solution was prepared as followed: A. Solid RNase A was dissolved to 10 mg cm^{-3} in 10 mmol dm^{-3} Tris-HCl pH 7.5, 15 mmol dm^{-3} NaCl. B. The contents were heated to 100°C for 20 min. C. The solution was cooled slowly to room temperature and then stored in aliquots at -20°C). Genomic DNA should be digested cleanly with a selection of restriction enzymes and be visible as a smear of restriction fragments from a few hundred base-pairs (bp) to several thousand after electrophoresis. The size of the DNA molecules was estimated by electrophoresis on the same agarose gel with lambda as a size marker (see section 4.2.10).

4.2.8 Transformation of competent *Escherichia coli* cells

Highly efficient competent *E. coli* SCS1 cells were provided by Dr. J. F. Hall in the Department. The procedure for preparation and storage of SCS1 cells is based on a method described by Nishimura *et al.* (1990).

For transformation, 0.1 cm^3 of the frozen SCS1 cells were thawed on ice, mixed immediately with 8 mm^3 (500 pg) of plasmid pSK4 containing the complete maize ABP cDNA (kindly provided by Dr. C. M. Lazarus in the University of Bristol), and incubated on ice for 30 min. The cells were then subjected to a heat pulse at 42°C for 1 min, then chilled on ice for 2 min, diluted 10-fold into prewarmed LB medium (10 g dm^{-3} bacto-tryptone, 5 g dm^{-3} bacto-yeast extract, 5 g dm^{-3} NaCl, pH 7.0) and shaken gently at 37°C for 1 hr to allow expression of antibiotic resistance. Samples (10 mm^3) were plated on agar plates containing 20 μg cm^{-3} of ampicillin. Transformation frequencies were calculated on the basis of colony counts after 20 hrs incubation at 37°C.

4.2.9 Isolation of plasmid DNA

Isolation of plasmid DNA from *E. coli* can be performed on either a large, "maxi-prep" scale to provide a stock of plasmid for longer-term use, or on a small or "mini-prep" scale to obtain sufficient material for cursory analysis. The isolation of plasmids is performed in essentially three stages. The bacterial cell wall is first weakened by the action of

lysozyme, and the cells are then lysed by EDTA and a detergent at high pH. Finally, the insoluble cell debris consisting of genomic DNA and protein is precipitated with high salt and centrifuged down, leaving the plasmid in solution.

4.2.9.1 Large-scale plasmid preparation

5 cm^3 bacto-nutrient broth (2.5 g dm^{-3}) was inoculated with *E. coli* containing the desired plasmid and incubated overnight at 37°C in an orbital shaking incubator. The culture contained the antibiotic ampicillin at a concentration of 20 μg cm^{-3}. The overnight culture was transferred into 1 dm^3 prewarmed LB medium and incubated under the same conditions until the A_{600} reached 0.7 when read on the spectrophotometer. At this stage, 10 cm^3 of 15 mg cm^{-3} chloramphenicol solution (in 100% ethanol) was added to the culture to amplify the replication of plasmid DNA but inhibit that of bacteria chromosomal DNA. The culture was kept under the same conditions for 12-16 hrs to allow the amplification of plasmid DNA.

Cells were harvested by centrifugation at 2000 *g* for 30 min in a JA-14 rotor in Beckman J 2-21 centrifuge at 4°C. Pellets were resuspended on ice in 12.5 cm^3 buffer containing 25% (w/v) sucrose and 50 mmol dm^{-3} Tris-HCl (pH 8.0) and transferred to a 250 cm^3 flask. 4 cm^3 of lysozyme solution, which contained 5 mmol dm^{-3} Tris-HCl (pH 8.0) and 10 mg cm^{-3} lysozyme (50,000 units mg^{-1} protein), was added, swirled and left on ice for 5 min. 20 cm^3 of 2% (v/v) Triton X-100 solution (from a fresh 10% stock) was then added and

swirled gently for 30 sec, left on ice for further 20 min followed by 30 sec gentle shaking.

The mixture was centrifuged at 25,000 *g* for 30 min at 4°C in a JA-20 rotor in Beckman J 2-21 centrifuge. 30 cm^3 of supernatant was transferred into a 150 cm^3 flask and supplemented with 1 g cm^{-3} CsCl. This was mixed gently until the CsCl was dissolved completely. An aliquot (0.75 cm^3) of ethidium bromide solution (10 mg cm^{-3}) was then added and mixed in. The mixture was left in the dark for 30 min.

The mixture was centrifuged at 2000 *g* for 30 min at 20°C in a JA-20 rotor in Beckman J 2-21 centrifuge. The supernatant was transferred to vertical rotor ultracentrifuge tubes through a syringe and 21 gauge needle. Samples were centrifuged at 300,000 *g* for 18 hrs at 20°C in a Europa 55M ultracentrifuge vertical rotor. Two bands could be visualized with a long-wave UV light. The upper band contained fragments of chromosomal DNA and also linear and open circle forms of plasmid, while the lower band contained supercoiled, covalently closed, circular plasmid.

To facilitate the withdrawal of the band, the centrifuge tube was pierced at the top with a 21 gauge needle. The bottom DNA band was removed by side puncture of the tube with a 21 gauge needle just below the band, and then was carefully drawn off into a syringe and transferred to a sterile test tube.

An equal volume of α-butanol saturated with TE buffer (pH 8.0) was added to the plasmid

solution and mixed gently to extract ethidium bromide. The upper phase was discarded. Fresh butanol was added to the DNA solution and this extraction was repeated until all pink colouration was removed (at least five times). The DNA solution was dialysed three times against 2 dm^3 of TE buffer (pH 8.0) at 4°C, using dialysis tubing prepared by twice autoclaving in 5% (w/v) sodium carbonate, 5 mmol dm^{-3} EDTA and storing at 4°C in sterile H_2O.

4.2.9.2 Small-scale plasmid preparation

1. 6 cm^3 overnight culture of *E. coli* was transferred and centrifuged at 15,000 *g* for 10 min.

2. The supernatant was discarded. The cell pellet was resuspended in 0.7 cm^3 of buffer (0.5 mol dm^{-3} EDTA, 8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50 mmol dm^{-3} Tris-HCl pH 8.0 plus 2 mg cm^{-3} freshly weighed lysozyme) and incubated on ice for 20 min.

3. The tubes were placed in a boiling water bath for 1 min and centrifuged at 13,000 rpm for 10 min in a microfuge.

4. The supernatants were transferred to fresh Eppendorf tubes and washed three times with phenol-chloroform (Section 4.2.5.2) and once with chloroform only.

5. The aqueous phase (top) was removed and the volume was estimated.

6. 1/10 volume (total) 3 mol dm^{-3} NaAc (pH 5.2) and 2.5 volume absolute ethanol (-20°C) was added and the sample was left overnight for DNA precipitation.

7. The DNA was spun down at 13,000 rpm in a microcentrifuge for 10 min and then was rinsed with 0.5 cm^3 70% ethanol.

8. The sample was centrifuged again. The supernatant was poured off and the precipitate was dried under vacuum.

9. The sample was resuspende in 50 mm^3 sterile water and 5 mm^3 of the suspension was electrophoresed in a 0.7% agarose gel for the estimation of yield (see section 4.2.10). The rest of the sample was kept at -20°C.

4.2.9.3 Plasmid minipreps with the Magic Minipreps™ DNA purification system

The Magic Minipreps DNA purification system (Promega Corporation, USA) provides a simple and reliable method for rapid isolation of plasmid DNA. The entire miniprep process can be completed in 15 min or less, with no organic extractions and ethanol precipitations. DNA is eluted from the Magic Minipreps mini-column in water or TE buffer, free of any salt or macromolecular contaminants. The purified plasmid can be used

directly for DNA sequencing, transcription *in vitro*, or restriction digestion without further manipulation.

1. 3 cm^3 of cell culture was centrifuged at 10,000 rpm in a JA-14 rotor in Beckman J 2-21 centrifuge. The cell pellet was resuspended in 200 mm^3 of Cell Resuspension Solution (50 mmol dm^{-3} Tris-HCl pH 7.5, 10 mmol dm^{-3} EDTA, 100 μg cm^{-3} RNase A). The resuspended cells were transferred to an Eppendorf tube.

2. 200 mm^3 of Cell Lysis Solution (0.2 mol dm^{-3} NaOH, 1% SDS) was added and the contents were mixed by inverting the tube several times. The cell suspension will clear almost immediately. If it does not, continue inverting until it clears.

3. 200 mm^3 of Neutralization Solution (2.55 mol dm^{-3} KOAc pH 4.8) was added and the contents were mixed by inverting the tube several times.

4. The tube was spun in a microcentrifuge at 13,000 rpm for 5 min.

5. The cleared supernatant was decanted to a new microcentrifuge tube.

6. 1 cm^3 of Magic Minipreps DNA Purification Resin was added and the contents were mixed by inverting the tube.

7. One Magic Minipreps mini-column was used for each miniprep. A 3 cm^3 disposable syringe barrel was attached to the luer-lok extension of each mini-column.

8. The Magic Minipreps DNA Purification Resin containing the bound DNA was decanted into the syringe barrel. The syringe plunger was inserted and the slurry was gently pushed into the mini-column with the syringe plunger.

9. The mini-column was washed with 2 cm^3 Column Washing Solution (200 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris-HCl pH 7.5, 5 mmol dm^{-3} EDTA, 50% (v/v) ethanol) by removing the mini-column from the syringe and taking up the solution in the syringe. The syringe was re-attached to the mini-column and the Column Wash Solution was gently pushed through the mini-column with the syringe plunger.

10. The mini-column was transferred to a microcentrifuge tube and spun in a microfuge for 20 sec for the resin to dry.

11. The mini-column was transferred to a new microcentrifuge tube.

12. 50 mm^3 of water or TE buffer preheated to 65-70°C was applied to the mini-column.

13. The tube containing the mini-column was spun for 20 sec in a microfuge. The mini-column was removed and discarded. Plasmid DNA may be stored in the microcentrifuge

tube at 4° or -20°C. Each Magic Miniprep DNA isolation will yield up to 10 μg of plasmid DNA.

4.2.10 Preparation of agarose gel for DNA separation

Agarose gels are nowadays much the most popular medium for the electrophoretic separation of medium- and large-sized nucleic acid (Serwer, 1981). The concentration of agarose gel depends upon the size of nucleic acids to be separated. An approximate guide is shown in Table 4.2.

Table 4.2 Agarose concentrations required to separate nucleic acids of various sizes

Agarose (%)	0.3	0.5	0.8	1.0	1.2	1.5	2.0
Linear nucleic acid size (kb)	1.0-70	0.7-45	0.4-20	0.3-10	0.2-8	0.2-6	0.1-5

1. The agarose was melted in 9/10 final volume distilled H_2O and 1/10 volume 10 x TBE buffer (0.89 mol dm^{-3} Tris-base, 0.89 mol dm^{-3} boric acid, 20 mmol dm^{-3} EDTA, pH 8.0).

2. The solution was cooled to 50°C and then ethidium bromide (10 mg cm^{-3} solution) was added to a final concentration of 0.5 μg cm^{-3}.

3. The solution was poured into the mould and the sample well-forming comb was immediately placed in position (Maniatis *et al.*, 1982).

4. When the gel had completely cooled and set (30-40 min), the comb was removed and the gel was placed in the electrophoresis apparatus.

5. Sufficient 0.5 x TBE buffer was added to fill the electrode chambers and to cover the gel to a depth of about 1 mm.

4.2.11 Restriction enzyme digestion

Restriction enzymes and their buffers were obtained from Promega Corporation, USA.

The required units of enzyme for each digest were obtained using the equation

$$\text{Units enzyme} = \frac{\mu\text{g of DNA x (final volume/20) x 4}}{\text{reaction time (hrs)}}$$

DNA was transferred to a microcentrifuge tube, the appropriate volume of 10 x digestion buffer (specific for each enzyme) and water were added and gently mixed with the pipette tip. The required units of the restriction enzymes were then added into the tube and mixed

again. The reaction mixture was incubated in a water bath at the appropriate temperature for the desired length of time. The reaction was stopped by adding 0.5 mol dm^{-3} EDTA (pH 7.5) to a final concentration of 10 mmol dm^{-3}. DNA was purified by extraction with phenol-chloroform (see section 4.2.5.2). and resuspended in sterile H_2O. The result of DNA digestion was analysed by gel electrophoresis (see section 4.2.10).

4.2.12 Recovery of DNA from agarose gels

The DNA contained within gel slices can be electro-eluted by placing the slices in a dialysis tube in an electric field. The DNA elutes from the gel and adheres to the dialysis tube wall, from which it can be freed by reversing the current for a short time. The buffer containing the DNA is removed and extracted with phenol-chloroform, precipitated using ethanol. 1% (w/v) agarose is prepared in TAE buffer (40 mmol dm^{-3} Tris-acetate, pH 7.8, 0.2 mmol dm^{-3} EDTA) which is suggested to yield better quality DNA.

1. Electrophoresis of the DNA on a gel containing 0.5 μg cm^{-3} ethidium bromide, as described in section 4.2.10, allowed visualisation of the DNA bands when placed on a UV trans-illuminator.

2. Gel slices containing the DNA fragments were conveniently excised with a single-edge razor blade, being careful not to damage the trans-illuminator surface.

3. A gel slice containing the DNA fragment was transferred into a dialysis tube which had been rinsed in distilled water. The tubing was prepared by boiling for 10 min in 50 mmol dm^{-3} sodium carbonate, 1 mmol dm^{-3} EDTA (pH 8.0) and stored at 4°C.

4. 50 volumes of TAE buffer per volume of gel slice was placed into the tube and the ends were clamped.

5. The tube was placed in the electrophoresis tank such that the long dimension of the agarose block was parallel with the dialysis tube walls and to the electrodes of the tank. Electrophoresis was carried out for 15-30 min at 150 volts until the fluorescing band of DNA could be seen to transfer from the gel.

6. Without disturbing the position of the tube in the tank, the current was reversed for the DNA to be released from the dialysis tube and enter the buffer solution; 60 sec is sufficient, otherwise the DNA may bind to the opposite wall of the tube.

7. The buffer containing the DNA was removed from the dialysis tube to a microcentrifuge tube and the dialysis tube was rinsed with 1 cm^3 TAE buffer. The two solutions were combined.

8. The DNA was purified by phenol-chloroform extraction and ethanol precipitation (see section 4.2.5.2).

4.2.13 DNA-DNA hybridization with non-radioactive probe

A non-radioactive DNA-DNA hybridization method was used in this work. This method was based on Boehringer Mannheim's protocol for the DIG DNA labelling and detection kit. DNA is labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate (Reinberg and Volelstein, 1983). The dUTP is linked via a spacer-arm to the steroid hapten digoxigenin (DIG-dUTP). After hybridization to the target DNA the hybrids are detected by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin-alkaline phosphatase conjugate) and subsequent enzyme-catalyzed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) or light reaction with the Lumi-Phos^{TM} 530.

The kit contains the following vials:

1. Unlabelled control DNA1 pBR328 DNA at 100 μg cm^{-3}, digested separately with *Bam* HI, *Bgl* I and *Hinf* I. The separate digests are combined in a ratio of 2:3:3. Sizes of the 16 pBR328 fragments: 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298 (2 x), 234 (2 x), 220 and 154 (2 x) bp.

2. Unlabelled control DNA 2 pBR328 at 200 μg cm^{-3}, linearized with *Bam* HI.

3. DNA dilution buffer Herring sperm DNA at 50 μg cm^{-3}, in Tris-HCl, 10 mmol dm^{-3};

EDTA, 1 mmol dm^{-3}; pH 8.0 (20°C).

4. Labelled control DNA 50 mm^3 linearized pBR328 DNA, labelled with digoxigenin according to the standard protocol containing 1 μg template DNA and approximately 260 ng synthesized labelled DNA.

5. Hexanucleotide mixture 10 x concentrated hexanucleotide reaction mixture.

6. dNTP labelling mixture 10 x concentrated dNTP labelling mixture containing dATP, 1 mmol dm^{-3}; dCTP, 1 mmol dm^{-3}; dGTP, 1 mmol dm^{-3}; dTTP, 0.65 mmol dm^{-3}; DIG-dUTP, 0.35 mmol dm^{-3}; pH 7.5 (20°C).

7. Klenow enzyme labelling grade Klenow enzyme (2 units mm^{-3}).

8. Anti-digoxigenin-AP conjugate Polyclonal sheep anti-digoxigenin Fab-fragments, conjugated to alkaline phosphatase, 750 units cm^{-3}.

9. NBT Nitroblue tetrazolium salt at 75 mg cm^{-3} in dimethylformamide, 70% (v/v).

10. X-phosphate 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt at 50 mg cm^{-3}, in dimethylformamide.

11. Blocking reagent supplied as powder.

The following solutions were used in labelling and detection:

1. Buffer 1 Maleic acid, 0.1 mol dm^{-3}; NaCl, 0.15 mol dm^{-3}; pH 7.5 (20°C), adjusted with concentrated NaOH, autoclaved.

2. Blocking stock solution Blocking reagent (vial 11) was dissolved in buffer 1 to a final concentration of 10% (w/v) with shaking and heating either on a heating block or in a microwave oven. This stock solution was autoclaved and stored at 4°C subsequently.

3. Buffer 2 Blocking stock solution, diluted 1:10 in buffer 1 (final concentration = 1% blocking reagent).

4. Buffer 3 Tris-HCl, 100 mmol dm^{-3}; NaCl, 100 mmol dm^{-3}; $MgCl_2$, 50 mmol dm^{-3}; pH 9.5 (20°C).

5. Buffer 4 Tris-HCl, 10 mmol dm^{-3}; EDTA, 1 mmol dm^{-3}; pH 8.0 (20°C).

6. Colour-substrate solution 45 mm^3 NBT-solution (vial 9) and 35 mm^3 X-phosphate-solution (vial 10) were added to 10 cm^3 buffer 3. This solution should be prepared freshly.

7. Lumi-Phos™ 530 Ready-to-use solution from Boehringer Mannheim.

8. Other solutions

EDTA, 0.2 mol dm^{-3}; pH 8.0 (20°C).

LiCl, 4 mol dm^{-3}.

Ethanol, 70%.

N-Lauroylsarcosine, 10% (w/v).

SDS, 10% (w/v).

20 x SSC: NaCl, 3 mol dm^{-3}; Na-citrate, 0.3 mol dm^{-3}; pH 7.0.

TE buffer: Tris-HCl, 10 mmol dm^{-3}; EDTA, 1 mmol dm^{-3}; pH 8.0 (20°C).

2 x SSC; SDS, 0.1% (w/v); sterile.

0.1 x SSC; SDS, 0.1% (w/v); sterile.

4.2.13.1 DNA labelling

The random primed DNA labelling method (Feinberg and Volelstein, 1983) allows efficient labelling of small (10 ng) and large (up to 3 μg) amounts of DNA per standard assay. Larger amounts can be labelled by scaling up of all components and volumes. Linear DNA is labelled more efficiently than circular and supercoiled DNA.

1. Purified DNA as supplied with the kit was used or the linearized DNA was purified as describe in section 4.2.11 and 4.2.12 and used for labelling.

2. DNA was denatured by heating in a boiling water bath (100°C) for 10 min and chilling quickly on ice.

3. The following was added to a microcentrifuge tube on ice:

1 μg of freshly denatured DNA, corresponding to 5 mm^3 control DNA (vial 2),

2 mm^3 hexanucleotide mixture (vial 5),

2 mm^3 dNTP labeolling mixture (vial 6), make up to 19 mm^3 with sterile redistilled water (control reaction requires 10 mm^3 sterile redistilled water), and add

1 mm^3 Klenow enzyme (vial 7).

4. The tube was centrifuged briefly and incubated overnight at 37°C.

5. The reaction was stoped by adding 2 mm^3 EDTA solution, 0.2 mol dm^{-3} (pH 8.0).

6. The labelled DNA was precipitated with 2.5 mm^3 LiCl, 4 mol dm^{-3}, and 75 mm^3 prechilled (-20°C) ethanol. The content was mixed well.

7. The sample was left for at least 30 min at -70°C or 2 hrs at -20°C.

8. The sample was spun at 13,000 rpm in a microfuge.

9. The pellet was washed with cold ethanol (70%), dried under vacuum and dissolved in

50 mm^3 TE buffer.

4.2.13.2 Dot blot

In dot blot, the transfer of denatured DNA is performed by spotting small volumes of DNA solutions onto nitrocellulose or nylon membrane. The stages are as follows:

1. Denature DNA by heating in a boiling water bath (100°C) for 10 min and chilling quickly on ice.

2. Spot the DNA solution, usually 1-5 mm^3, onto a nylon membrane (Hybond N, Amersham) and blow warm air on the membrane to minimize spot spreading.

3. Fix DNA onto nylon membrane by UV crosslinking for 3 min.

4. Treat the membrane as below in Southern blot.

4.2.13.3. Southern blot

1. At the end of the enzyme digestion, 1/5 sample volume of 6 x concentrated gel-loading buffer (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) was added and the fragments of DNA was separated by electrophoresis through an agarose

gel (see section 4.2.10 and 4.2.11).

2. After electrophoresis was completed, the gel was photographed. A transparent ruler was placed alongside the gel so that the distance that any band of DNA had migrated could be read directly from the photographic image.

3. The gel was transferred to a glass baking dish and any unused areas of the gel were trimed away with a razor blade. The bottom left-hand corner of the gel was cut off; this served to orient the gel during the succeeding operations.

4. The gel was soaked for 10 min in several volumes of 0.2 N HCl and then rinsed briefly with deionized water.

5. The DNA was denatured by soaking the gel for 45 min in several volumes of 1.5 mol dm^{-3} NaCl, 0.5 N NaOH with constant but gentle agitation on a rotary platform shaker.

6. The gel was rinsed briefly in deionized water, and then neutralized by soaking for 30 min in several volumes of a solution of 1 mol dm^{-3} Tris-HCl (pH 7.4), 1.5 mol dm^{-3} NaCl at room temperature with constant and gentle agitation. The neutralization solution was changed and the gel was soaked for a further 15 min. (While the gel was in the neutralization solution, the nylon membrane was prepared as follows: A. A piece of membrane about 1 mm larger than the gel in both dimensions was cut. Gloves and blunt-

ended forceps were used to handle the membrane. B. The membrane was floated on the surface of a dish of deionized water until it became completely wet from beneath, and then the membrane was immersed in transfer buffer (10 x SSC) for at least 5 min. Using a clean scalpel blade, a corner from the membrane was cut to match the corner cut from the gel.)

7. The gel was removed from the neutralization solution and inverted so that its underside was now uppermost. The inverted gel was placed on the support so that it was centered on the wet 3MM papers. It is important that there are no air bubbles between the 3MM paper and the gel.

8. The gel was surrounded, but not covered, with cling film.

9. The nylon membrane was placed on top of the gel so that the cut corners were aligned. One edge of the membrane filter extended just over the edge of the line of slots at the top of the gel. (Do not move the filter once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the filter and the gel.)

10. Two pieces of 3MM paper (cut to exactly the same size as the gel) were wetted in 2 x SSC and placed on top of the wet nylon filter. Any air bubbles were smoothed out with a glass rod.

11. A stack of paper towels (5-8 cm high) just smaller than the 3MM papers was placed

on the 3MM papers. A glass plate was placed on top of the stack and was weighed down with a 500-g weight.

12. The transfer of DNA was allowed to proceed for 8-24 hrs. As the paper towels became wet, they were replaced.

13. The paper towels and the 3MM papers above the gel were removed. The gel and the nylon filter were turned over and laid on a dry sheet of 3MM paper (gel side up). The positions of the gel slots on the filter were marked with a very-soft-lead pencil or a ballpoint pen.

14. The gel was peeled from the filter and discarded. The filter was soaked in 6 x SSC for 5 min at room temperature so that any pieces of agarose sticking to the filter were removed.

15. The filter was removed from the 6 x SSC and excess fluid was allowed to drain away. The filter was placed on a paper towel to dry for at least 30 min at room temperature.

16. The DNA was fixed by exposing the side of the membrane carrying the DNA to UV for 3 min.

4.2.13.4 Hybridization

Hybridization buffer: 5 x SSC; blocking reagent, 1% (w/v) (added from 10% sterile blocking stock solution); *N*-lauroylsarcosine, 0.1% (w/v); SDS, 0.02% (w/v). The hybridization buffer can be stored frozen at -20°C.

Control

1. A dilution series of the unlabelled control DNA (vial 1) was made in dilution buffer (vial 3) from 10 pg mm^{-3} to 10 fg mm^{-3}.

2a. Dot blot: DNA was denatured by heating in a boiling water bath and chilling quickly on ice. 1 mm^3 of the diluted control or heterologous DNA (vial 3) was spotted onto the nylon membrane.

2b. Southern blot: The diluted control DNA (100 pg - 1 pg per lane) was loaded onto an agarose gel. The fragments were separated and a Southern transfer (as above) to membrane was subsequently performed.

3. DNA was bound to nylon membrane by UV crosslinking for 3 min.

4. Filters were pre-hybridised in a sealed plastic bag or box with at least 20 cm^3 hybridization buffer per 100 cm^2 of filter at 68°C for at least 1 hr. The solution was distributed from time to time. The filters should not be allowed to dry out between pre-

hybridization and hybridization.

5. The solution was replaced by about 2.5 cm^3 per 100 cm^2 filter of hybridization buffer containing 5 mm^3 (26 ng) of freshly denatured labelled control DNA (vial 4) per cm^3.

6. The filters were incubated for at least 6 hrs at 68°C. The solution was re-distributed occasionally.

7. Filters were washed 2 x 5 min at room temperature with at least 50 cm^3 of 2 x SSC; SDS, 0.1% (w/v), per 100 cm^2 filter and 2 x 15 min at 68°C with 0.1 x SSC; SDS, 0.1% (w/v).

8. Filters could then be used directly for detection of hybridized DNA or stored air-dried for later detection.

Standard assay

1. The DNA to be probed was transferred onto a nylon membrane by dot blot or Southern blot.

2. The DNA probe was labelled according to the standard assay procedure as above.

3. Filters were pre-hybridized as described for the control reaction. The filters should not be allowed to dry out between pre-hybridization and hybridization.

4. The pre-hybridization solution was replaced by about 2.5 cm^3 per 100 cm^2 filter of hybridization solution containing freshly denatured probe DNA. Usually 10-100 ng of labelled DNA per cm^3 hybridization solution were used. About 2.5 cm^3 of hybridization solution per 100 cm^2 of filter were required.

5. The filters were hybridized and washed as described for the control reaction.

6. The hybridization solution containing labelled DNA could be stored at -20°C and reused several times. Immediately before use, the probe was re-denatured by heating the hybridization solution at 95°C for 10 min. This step also re-dissolved any precipitate, which might have formed during storage.

7. The filters could then be used directly for detection of hybridized DNA or stored air-dried.

4.2.13.5 Immuno-detection

Control and standard assay for immuno-colourimetric detection

All the following incubations are performed at room temperature and except for the colour reaction with shaking or mixing. The volumes of the solutions are calculated for a filter size of 100 cm^2 and should be adjusted to other filter sizes.

1. Filters were washed briefly (1 min) in buffer 1.

2. The filters were then incubated for 30 min with about 100 cm^3 buffer 2.

3. Antibody conjugate (vial 8) was diluted to 150 munit cm^{-3} (1:5000) in buffer 2.

4. The filters were incubated for 30 min with about 20 cm^3 of diluted antibody-conjugate solution.

5. Unbound antibody-conjugate was removed by washing the filters 2 x 15 min with 100 cm^3 of buffer 1.

6. The filters were equilibrated for 2 min with 20 cm^3 of buffer 3.

7. The filters were incubated with ca. 10 cm^3 colour solution sealed in a plastic bag or in a suitable box in the dark. The colour precipitate started to form within a few minutes and the reaction was usually complete after 16 hrs. (Do not shake or mix while colour is developing.)

8. When the spots or bands were detected, the reaction was stopped by washing the membranes for 5 min with 50 cm^3 of buffer 4.

9. After the results were documented, the filters could then be dried at room temperature and stored.

Control and standard assay for immuno-chemiluminescent detection

1. Experiment was performed as in immuno-colourimetric detection steps 1-6.

2. 2-5 cm^3 of Lumi-Phos™ 530 was added into the center of a petri dish. Using blunt-end forceps, the filter was repeatedly passed through the substrate solution until it was thoroughly wetted.

3. The membrane was removed from the Lumi-Phos™ 530 solution and any excess liquid was allowed to drip off. The filter should not be allowed to dry.

4. The damp filter was covered by enclosing it in a hybridization bag.

5. The bag was covered with aluminium foil and incubated at 37°C for 30 min to allow light emission to reach a steady state.

6. In a darkroom, the sealed filter was exposed to X-ray film. Following the initial exposure time (e.g. 15 min) testing, shorter or longer exposure times could be used to obtain the optimal signal intensity. After the 30 min incubation at 37°C (step 5), the light emission remained constant for approximately 24 hrs. Multiple exposures from a single blot could be obtained during this time.

4.3 Results

4.3.1 Solubilization and partial purification of membrane-bound auxin binding protein

4.3.1.1 Characterization

Specific binding of IAA was found in membrane particle preparations from sugar beet seedlings. The binding assay was based on a method described by Hertel *et al.* (1972). Binding of labelled IAA (10^{-9} mol dm^{-3}) was carried out with different concentrations of unlabelled IAA (from 0 to 10^{-4} mol dm^{-3}) and a Scatchard plot was constructed from this experimental data (Fig. 4-1). A dissociation constant (Kd) of 2.15 x 10^{-6} mol dm^{-3} was obtained with site concentration of 68.2 fmoles mg^{-1} membrane protein.

To obtain sufficient purified ABP, a large amount of sugar beet seedlings are needed. 3.4 Kg of maize seedlings were extracted and used in the procedures of solubilization and purification by Shimomura *et al.* (1986). However, the final yield of their purified ABP

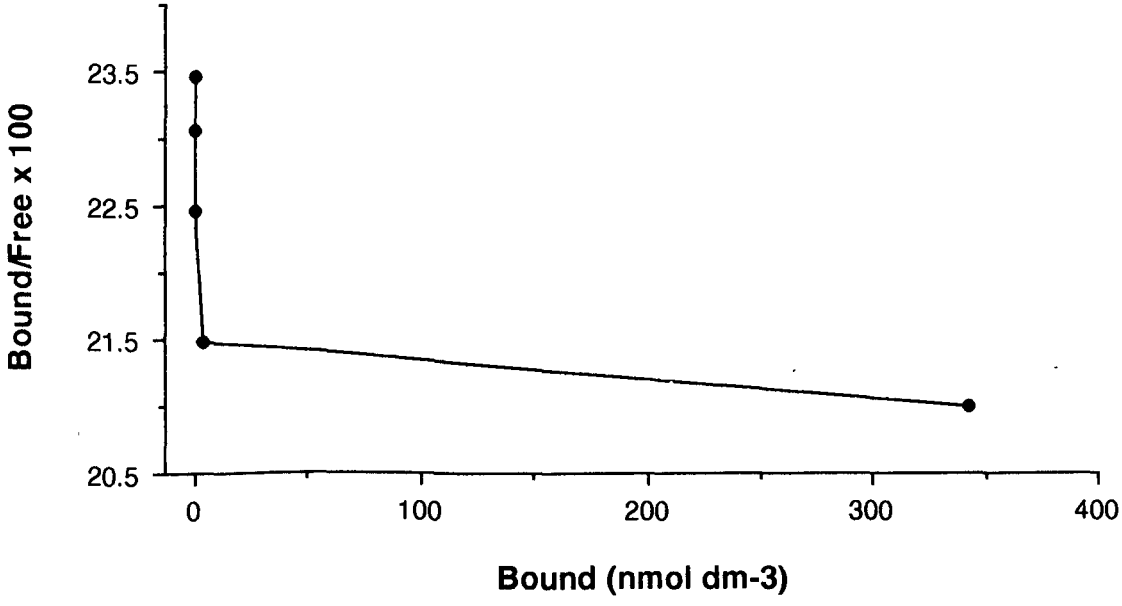


Fig. 4-1 Scatchard plot of IAA binding to membranes from sugar beet seedling leaf

was only 6% of the total present in the membranes. On the other hand, it normally takes five hours to complete the procedures of homogenization and solubilization. Cutting kilos of seedlings will certainly take one person numerous hours to do. Therefore, the specific IAA binding of fresh and frozen sugar beet seedlings was determined, in order to assess whether suitable amounts of material could be accumulated and stored at -20°C.

Samples of the sugar beet seedlings were frozen with liquid nitrogen and left at -70°C for two days. These frozed samples, together with fresh samples, were extracted, as described in section 4.2.2. Two-point assays were carried out as described in section 3.2.3. A concentration of 10^{-9} mol dm^{-3} ^{3}H-IAA (Specific activity 999 GBq $mmol^{-1}$) was added to each sample. The results, which are shown in Table 4.3, indicate a substantial loss of specfic binding of ^{3}H-IAA in the frozen seedlings.

Table 4.3 Specific IAA binding to membranes from fresh and frozen sugar beet seedlings

Seedlings	Specific ^{3}H-IAA binding (dpm g^{-1} fr.wt)	% of specific binding
fresh	5384.4	100
frozen	1733.8	32

Thus, it seems unrealistic to get a large amount of plant material by using frozen instead

of fresh sugar beet seedlings to avoid a long preparing period before the procedures of extraction and solubilization.

4.3.1.2 Solubilization

Pre-packed, disposable Pharmacia columns PD-10 containing Sephadex G-25 were initially calibrated for their void volumes by loading different amount of resuspension buffer containing both blue dextran 2000 and 10^{-9} mol dm^{-3} ^{3}H-IAA. The complete elution profiles were run as described in Dohrmann and Ray (1976), to determine the proper cut-off point to avoid overlap of the void peak with free ^{3}H-IAA. Table 4.4 shows the results of the test.

Table 4.4 Determination of the void volume of column PD-10 (Sephadex G-25) (Eluate was collected immediately after sample loading.)

Sample loading volume (cm^3)	Blue dextran initially present at (cm^3)	Blue dextran absent by (cm^3)	free ^{3}H-IAA first detected at (cm^3)
1.00	2.25	7.75	7.00
0.50	2.50	6.00	7.00
0.25	2.75	4.50	7.00

Since Sephadex G-25 has a resolution range of 1000-5000 daltons molecular weight, proteins (e.g. ABP) and other larger molecules of the sample should emerge with the excluded volume of the column. However, the maximum amount of loading sample has to be restricted to no more than 0.5 cm^3 because an overlap of the peak of ^{3}H-IAA and large protein molecules appeared when loaded 1.0 cm^3 of the sample. Therefore, using columns PD-10 containing Sephadex G-25 to measure the binding of solubilized extracts, the proper collecting period should be from 2.5 cm^3 to 7.0 cm^3.

Two methods have been reported for the preparation of solubilized ABP, i. e. extraction of intact membranes with a non-ionic detergent, Triton X-100 (Ray *et al.*, 1977; Cross *et al.*, 1978) and extraction of acetone-washed membranes without detergent (Venis, 1977). These two methods were compared in parallel on our membrane preparations. Binding of ^{3}H-IAA of solubilized extracts were measured with PD-10 Sephadex G-25M columns. Table 4.5 indicates that similar percentage of ABP can be solubilized with either of the methods. Since the concentration of solubilized extract can be managed more easily using the acetone-treatment method, by dissolving the pelleted washed membranes in a desired volume of assay buffer, this method was chosen for further study.

Although the use of PD-10 columns (Sephadex G-25) is a valid procedure for specific binding of solubilized ABP, the differences in bed volume, void volume and efficiency of resolution of each column are variable and cannot be neglected (data not shown). Using the Centrifree™ MPS-1 micropartition system to separate free ^{3}H-IAA from bound ABP after

Table 4.5 Comparison of solubilization methods

Treatment	Binding of ^{3}H-IAA (dpm)			% of specific
	total	non-specific	specific	binding
untreated membrane	53735.1 ±759.8	49085.3 ±66.2	4649.8	100
+ Triton X-100	9705.4 ±261.0	7932.1 ±150.0	1773.3	38.1
+ acetone	9317.6 ±232.6	7675.6 ±143.3	1642.0	35.3

solubilization by acetone precipitation gave higher recovery than using PD-10 columns (Table 4.6).

As shown in Table 4.6, 35% and 41% of solubilized ABP were detected by PD-10 column and Centrifree™ MPS-1 procedures, respectively. Therefore, Centrifree™ MPS-1 micropartition system was used in further studies on solubilization and purification. In contrast with gel filtration, Centrifree™ MPS-1 micropartition is a rapid equilibrium method which provides improved accuracy when measuring the free ligand concentration. Meanwhile, multiple samples are conveniently handled under identical conditions, with maximum efficiency.

Table 4.6 Comparison of detection techniques for solubilized auxin binding protein

Technique	Specific binding of ^{3}H-IAA (pmoles mg^{-1} protein)	% of specific binding
membrane-associated binding	2.25	100
Sephadex G-25 PD-10 column	0.79	35
Centrifree™ MPS-1 micropartition	0.92	41

4.3.1.3 Purification

An acetone-solubilized preparation was used for the purification of auxin binding protein. The purification included chromatographies on DEAE-Sephacel ion-exchange column, NAA-linked AH-Separose 4B affinity column and Sephadex G-200 gel filtration column. For the detection of auxin binding in partially purified fractions, ^{14}C-NAA and non-radioactive NAA were used for their stability, though IAA was used for the detection of binding in non-solubilized and solubilized preparations.

Ion-exchange chromatography has been used extensively in the purification of receptors.

The optimal pH of membrane-associated auxin binding proteins was reported to be lower than pH 6.5 (Libbenga *et al.*, 1987). It was therefore expected that ABP will adsorb to the anion exchanger DEAE-Sephacel at neutral pH and could be eluted by salt solution. A non-gradient salt solution (0.3 mol dm^{-3} NaCl) was used to desorb the proteins bound to the column. The elution profile is shown in Fig. 4-2. Although overall purification is approximately five-fold, the recovery was only 6.4% and over 90% of specific binding was lost in the process of purification (Table 4.7). No specific binding was detected in the non-adsorbed fractions. 2,4-D-linked AH-Sepharose 4B chromatography was successfully used in partial purification of sABP from tobacco cells in this laboratory (Bailey *et al.*, 1985; Barker and Bailey, 1988). Following the same principle, NAA-linked AH-Sepharose 4B column was prepared as described in section 4.2.4. To check whether NAA was able to be

Table 4.7 Purification of auxin binding protein by ion-exchange chromatography

Sample	Total protein (mg)	Specific binding (pmoles ^{14}C-NAA)	Specific activity (pmoles mg^{-1})	Yield (%)
solubilized membrane extract	63	344.9	5.5	100
DEAE-Sephacel column	0.84	22.2	26.5	6.4

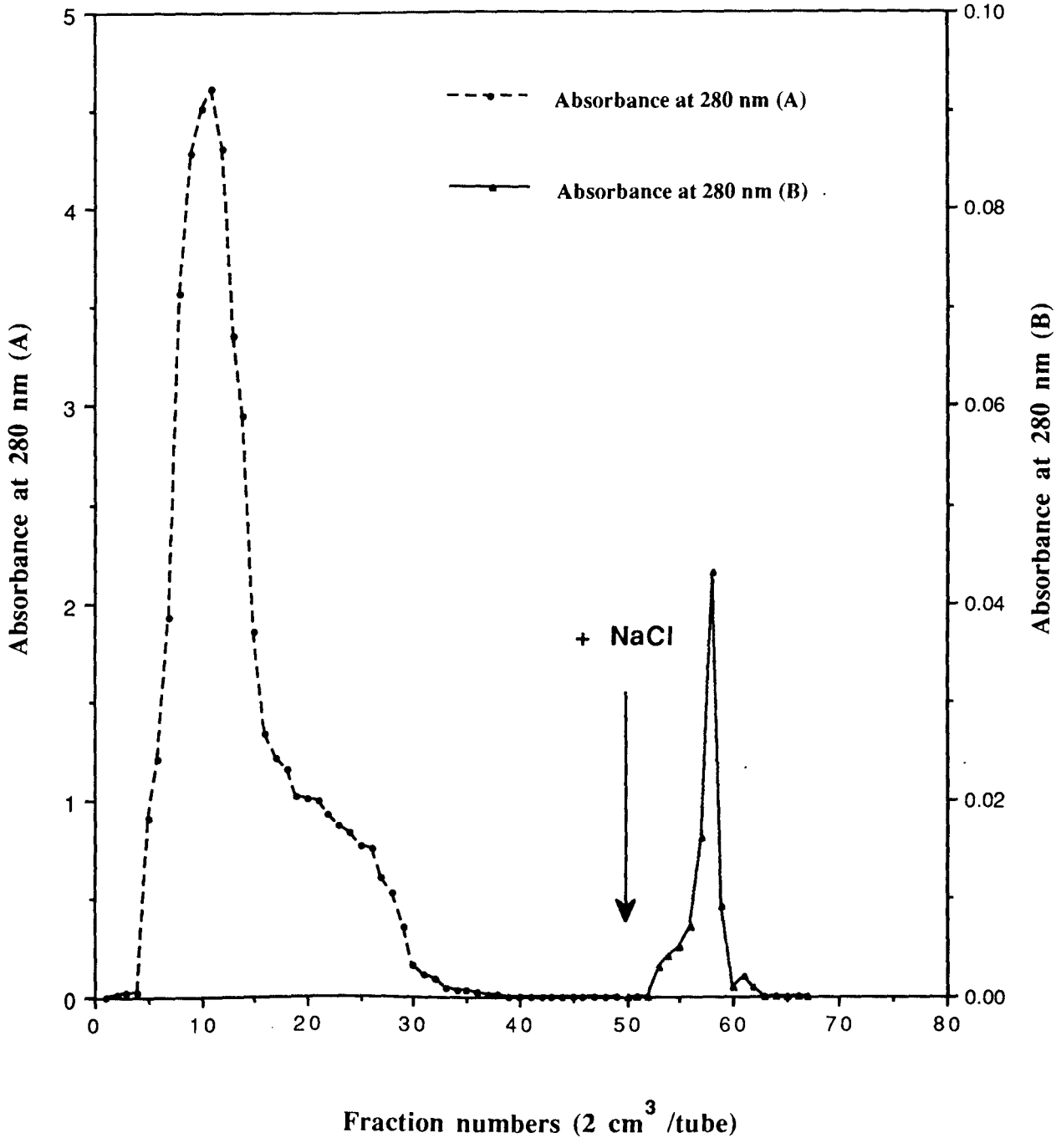


Fig. 4-2 DEAE-Sephacel chromatography of the solubilized extract of the acetone-washed membranes. The arrow indicates where 0.3 mol dm^{-3} NaCl was applied to the column

coupled to AH-Sepharose 4B, radioactive NAA was first tried as coupling ligand. 2 x 10^{-7} mol dm^{-3} ^{14}C-NAA and 5 cm^3 AH-Sepharose 4B were used. At the end of coupling procedure 1.13 x 10^{-8} mol dm^{-3} ^{14}C-NAA could be detected in ^{14}C-NAA-linked Sepharose conjugate. This result has confirmed the validity of the coupling technique.

Non-radioactive NAA was then used in creating a NAA-linked AH-Sepharose 4B column. This affinity chromatographic step has previously been found effective in removing a large portion of other proteins (Shimomura *et al.*, 1986). A solubilized preparation was applied to the column. After washing with 0.5 mol dm^{-3} NaCl, ABP was desorbed in the presence of NAA. A highly purified ABP preparation could be obtained. As shown in Table 4.8, the

Table 4.8 Purification of auxin binding protein by affinity chromatography

Sample	Total protein (mg)	Specific binding (pmoles C^{14}-NAA)	Specific activity (pmoles mg^{-1})	Yield (%)
solubilized membrane extract	86	482.5	5.6	100
NAA-linked AH-Sephorose 4B	0.037	6.3	170.5	1.3

specific activity (pmoles mg^{-1} protein) was increased by thirty times after affinity chromatography. However, the yield of ABP was very lower with recovery of only 1.3%. This high degree of loss was thought to be caused to large extent by gradual leakage of the

protein from the column at a high concentration of NaCl in the pre-desorption washing. It was indeed found that a high protein concentration was present in the eluate of this washing. When this pre-desorption eluate was concentrated to a single fraction and used in binding assay, 46% of the binding activity was regained. The remaining 52.7% was lost during the purification and it is assumed that it was inactivated or degraded in the pre-desorption washing and/or NAA-desorption washing.

Gel filtration techniques have been widely and successfully used for partial purification of ABP or sABP (Venis, 1977; Van der Linde *et al.*, 1984; Napier *et al.*, 1988; Shimomura *et al.*, 1986). A solubilized membrane preparation in a total volume of 3.5 cm^3 was applied to a Sephadex G-200 column. The elution profile is shown in Fig. 4-3. Fractions of 5 cm^3 were collected for the estimation of protein content. Each of the five successive fractions with total volume of 25 cm^3 were pooled and concentrated. A total of 16 such pooled fractions were obtained from the eluate and a binding assay was conducted on each of them. As indicated in Fig. 4-3, specific NAA binding was detected in pooled fractions 11 and 12. The total specific NAA binding in these fractions (=R-fraction) was 0.605 pmole mg^{-1} protein, which represented only 10.8% of the total present in the solubilized preparations (5.58 pmoles mg^{-1} protein). Although NAA binding could be observed after gel filtration, the ABP-containing R-fraction should not be considered to be exclusively ABP as it is likely to be contaminated by other proteins.

Although DEAE-Sephacel, NAA-linked AH-Sepharose 4B and Sephadex G-200 all

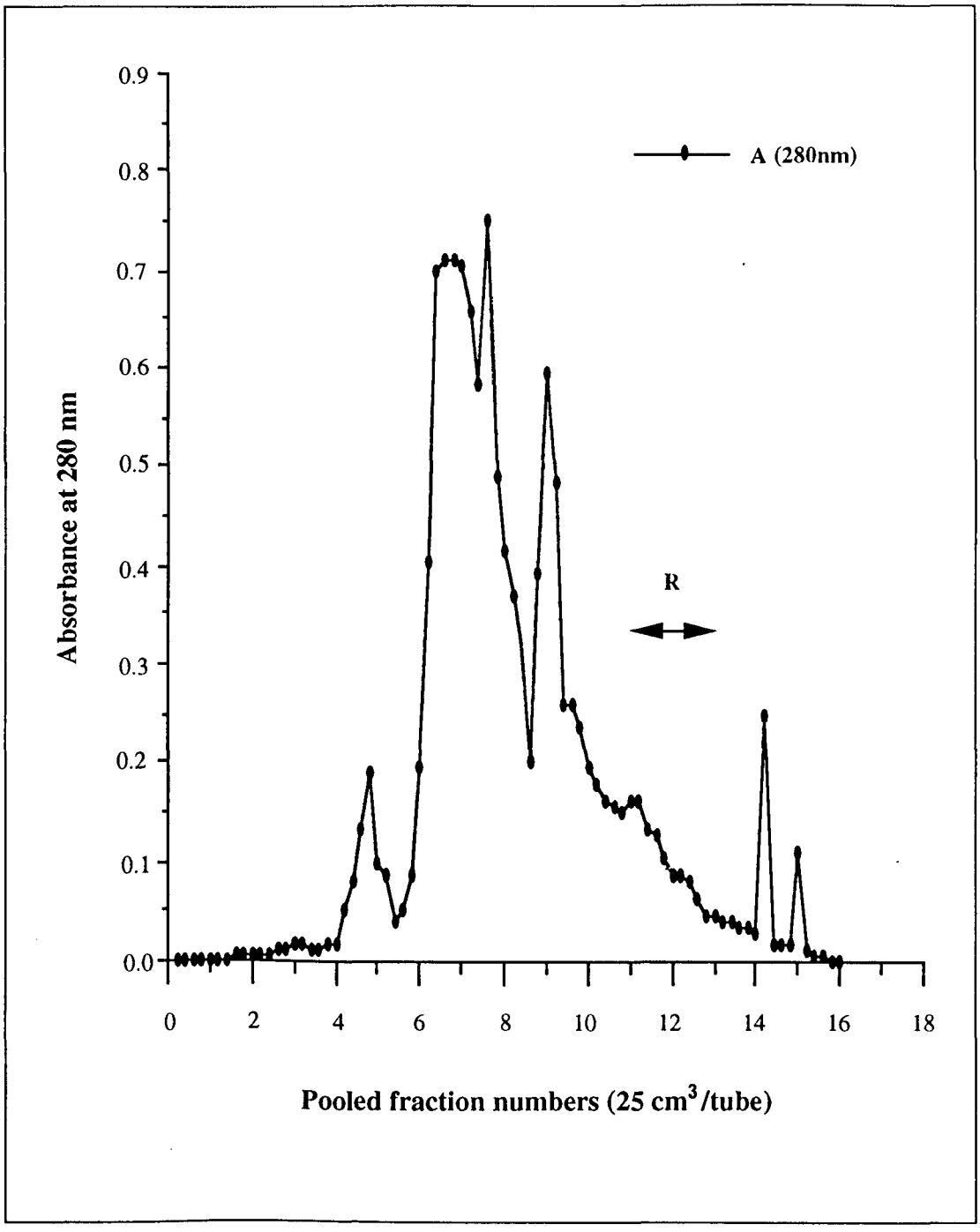


Fig. 4-3 Elution profile of solubilized membranes on Sephadex G-200. The R indicates pooled fractions containing specific NAA-binding

produced partially purified ABP, the considerably low recovery after each single purification step made the further purification by coupling one technique to another unlikely to be successful. Indeed, when the post-DEAE-Sephacel fraction was applied to a NAA-linked AH-Sepharose 4B column, no binding activity could be detected in the eluate washed off by NaCl plus NAA. Since the poor detectability results mainly from the low abundancy of ABP in our material, it seems difficult to expect the same success of ABP purification in sugar beet as in maize.

4.3.2 Isolation of ABP gene

4.3.2.1 Preparation of genomic DNA from maize and sugar beet cells

Isolation of genomic DNA from maize and sugar beet cells by phenol-chloroform extraction was described in section 4.2.5.2. DNA concentrations were measured by ultraviolet absorbance spectrophotometry (see section 4.2.6).

Table 4-9 Concentrations of DNA obtained from genomic DNA preparations

Sample	Absorbance		A_{260}/A_{280}	DNA (μg mm^3)
	A_{260}	A_{280}		
Maize	0.471	0.239	1.971	4.71
Sugar beet	0.207	0.106	1.953	2.07

The amount of ultraviolet radiation absorbed by a solution of DNA was directly proportional to the amount of DNA in the sample. Since the ratios of the absorbances at 260nm and 280nm (A_{260}/A_{280}) were higher than 1.8 in maize and sugar beet, the purity of both DNA samples was qualified for further experiments. As shown in Fig. 4-4, the total DNA samples, with and without pre-treatment with RNase, were clearly visible on an 0.7% agarose gel.

4.3.2.2 Mapping and isolation of the complete maize ABP cDNA fragment from agarose gel

The complete maize ABP cDNA was a gift from Dr. C. M. Lazarus of the University of Bristol. Plasmid pSK4 has the complete maize ABP cDNA plus flanking fragments of λ*gt*11 cloned as a *kpn*I-*Sst*I fragment. This plasmid is cloned in pUBS1 which is basically Bluescript from which the M13 intergenic region has been deleted, i.e. pUC with a Bluescript polylinker. The best way to prepare a probe was to digest plasmid pSK4 with *Pvu*II (C. M. Lazarus, personal communication). As shown in Fig. 4-5, this generates 5 bands on the gel, the fourth of which is the cDNA insert. This is 800bp in length, and extends from the 5'-untranslated region to beyond the poly(A) tail, a few bases into λ*gt*11 sequence.

Large amounts of plasmid DNA were isolated as described in section 4.2.9. The purified

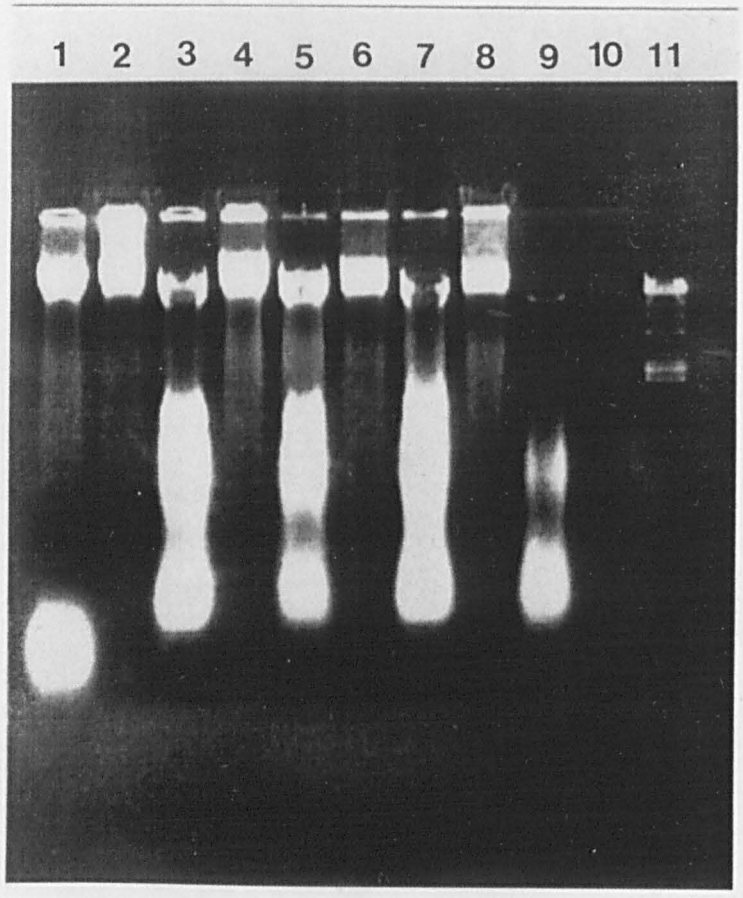


Fig. 4-4 Total DNA isolation from maize and sugar beet cells using phenol-chloroform extraction.

DNA samples with and without pre-treatment by RNase were run on a 0.7% agarose gel. Lanes 1,2: maize (4 μg/lane); Lanes 3-8: sugar beet (4 μg/lane); Lanes 9,10: sugar beet (1 μg/lane); Lanes 1,3,5,7,9: - RNase; Lanes 2,4,6,8,10: + RNase; Lane 11: 500 ng of Lambda *Hind* III markers.

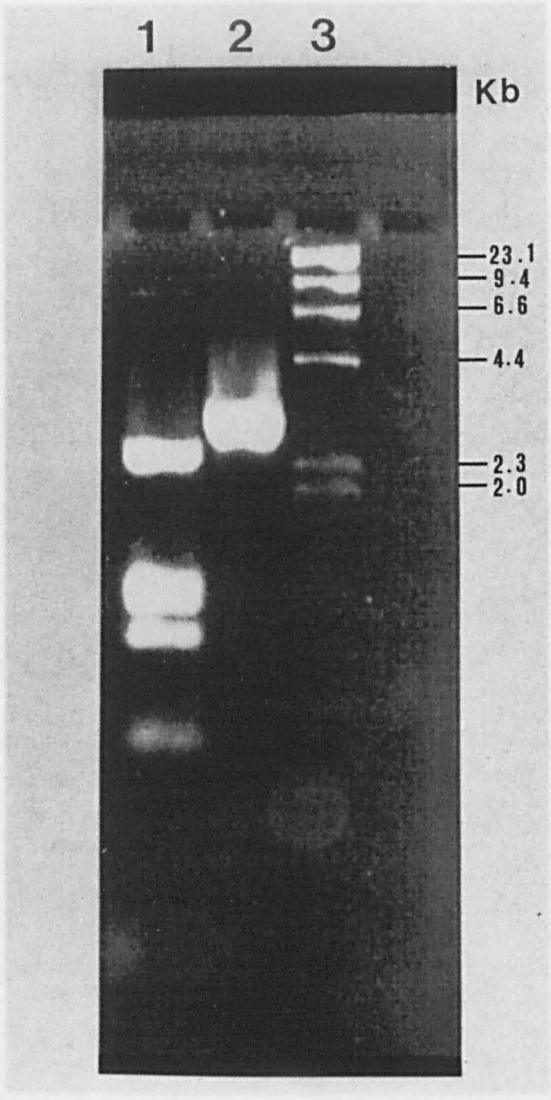


Fig. 4-5 Digestion of plasmid pSK4 with *Pvu*II.

Samples were run on a 0.8% agarose gel. Lane 1: 3 μg plasmid pSK4 was digested with *Pvu*II; Lane 2: 3 μg uncut plasmid pSK4; Lane 3: 1 μg of Lambda *Hind* III markers.

plasmid pSK4 was linearised with the restriction enzyme, *Pvu*II. Before labelling for use as a probe (see section 4.2.13.1), the complete maize ABP cDNA insert should be obtained from agarose gel. Several methods for retrieval of DNA from agarose gels have been developed (Towner, 1991) and are based either on physical separation or electroelution. Electroelution of DNA into a dialysis tube is the method used in our laboratory and it gives a good recovery. Plasmid pSK4 was digested with *Pvu*II and samples were run on 1% agarose gel (TAE buffer) containing ethidium bromide. Fig. 4-6A shows the result of this electrophoresis, in which the fourth band, which contains the ABP gene, is arrowed. This region of the gel was then excised and removed from the whole gel block under the observation with UV-transparent apparatus. Fig. 4-6B shows the same gel after excision of the ABP-containing band. The buffer containing the DNA was extracted with phenol-chloroform and precipitated with NaAc and ethanol (see section 4.2.12). The purified ABP cDNA was ready for labelling for use as a probe.

4.3.2.3 ABP cDNA probe labelling

The isolation, structural analysis and functional analysis of genes all involve the use of labelled nucleic acid. The choice of label for a particular application depends on two major parameters: the required resolution and the required sensitivity. In filter hybridization experiments, sensitivity is often the over-riding factor and at present, less widely used but more convenient non-radioactive labels claim equivalent sensitivity to ^{32}P (Pollard-Knight, 1990). Random primed DNA labelling with digoxigenin-dUTP and detection of hybrids by enzyme immunoassay were described in section 4.2.13. Fig. 4-7 shows direct immuno-

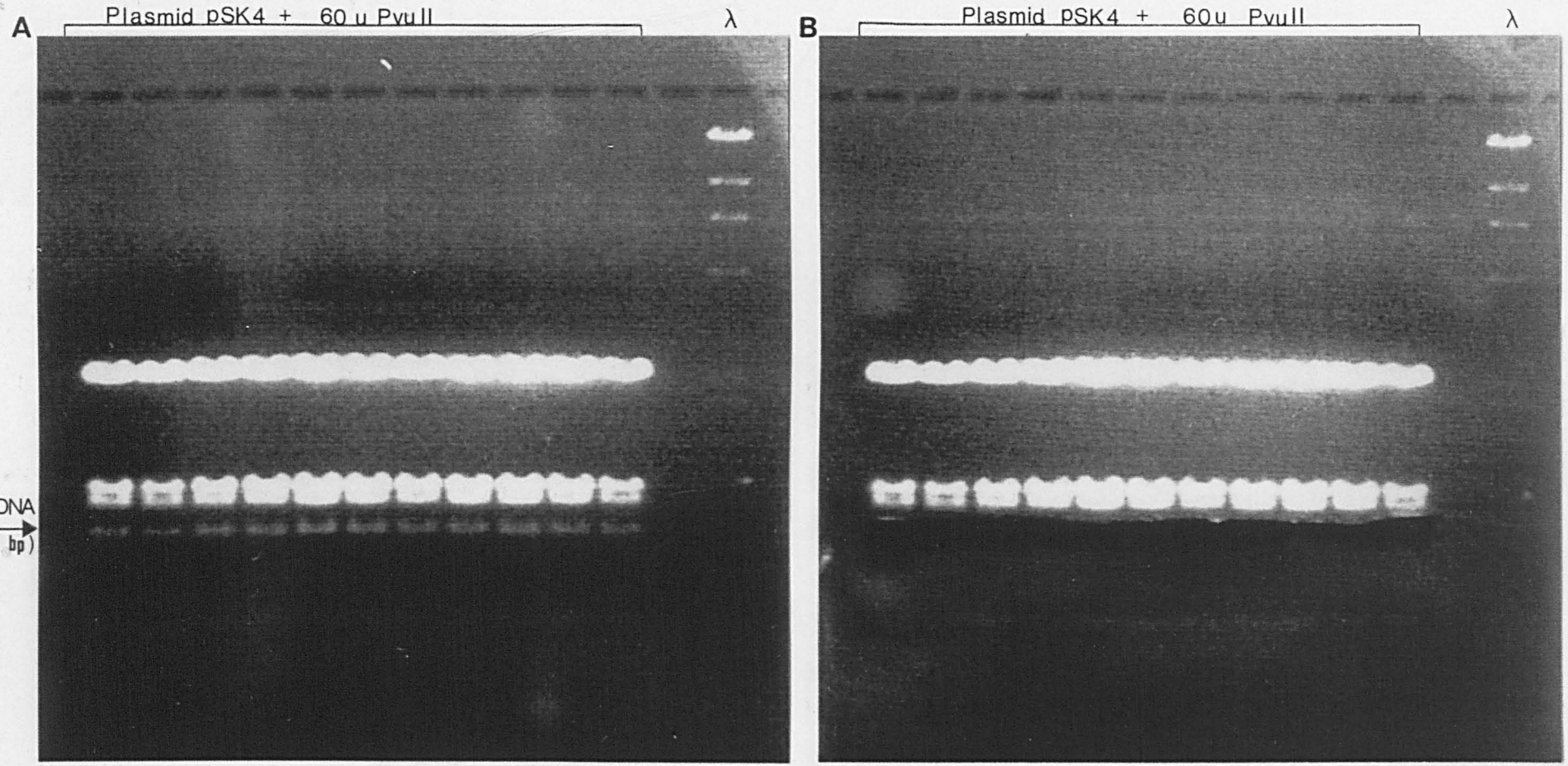


Fig. 4-6 Use of electro-elution to purify ABP cDNA

A: Plasmid pSK4 (2 μg per lane) was digested with *Pvu*II and resolved on a 1% agarose gel (TAE buffer). The 4th band, containing ABP gene, is arrowed.

B: Gel slices containing ABP cDNA were excised with a single-edge razor blade and removed from the gel block under the observation with UV-transparant apparatus. The remainder of the gel after removal of the ABP containing cDNA band, is shown.

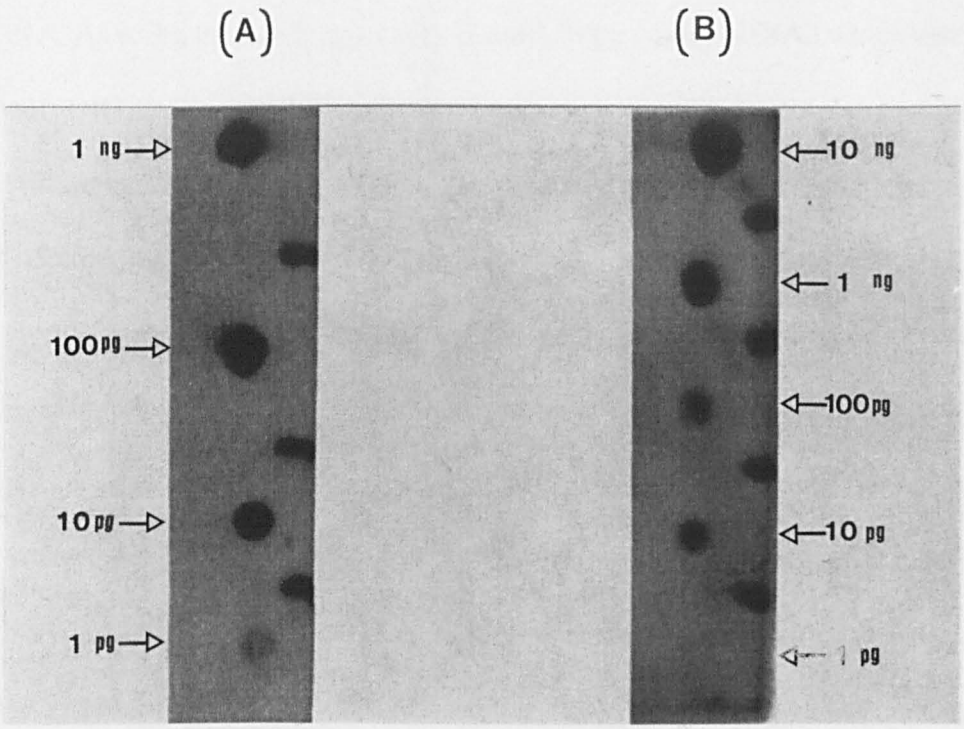


Fig. 4-7 Comparison of the non-radioactive labelled DNA generated with the labelled control-DNA by direct immuno-colourimetric detection.

A. Standard labelled control-DNA
B. Non-radioactive labelled control-DNA2

colourimetric detection of non-radioactive labelled control-DNA2 and standard labelled control-DNA. As using for labelling quality control, 10pg control-DNA2 which was freshly labelled with DIG as described in section 4.2.13.1, was detectable in a dot blot after 1 hr colour development. This indicated that the procedure for DIG labelling is efficient. Amounts of homologous control DNA1 (10ng - 10fg) were used as the labelling quality control, and maize genomic DNA (20μg to 1ng) were spotted on Hybond-N membranes. Freshly denaturated non-radioactive labelled control-DNA2 (26ng) and maize ABP cDNA (60ng) were added to the hybridization solution for DNA-DNA hybridization, respectively. Fig. 4-8 shows the immuno-chemiluminescent detection of hybridization homologous control-DNA1 and maize genomic DNA with non-radioactive labelled control-DNA2 and maize ABP cDNA probes respectively. Using the unlabelled control-DNA2 as a labelling sensitivity control, the concentration of 0.1 pg homologous control-DNA1 was to be detected. Although chemiluminescent and colourimetric detection have inherent advantages, using Lum-Phos^{TM} 530 for the immuno-chemiluminescent detection results in higher sensitive detection than colourimetric detection. Meanwhile, the use of Lumi-Phos 530 allows results to be recorded on X-ray film and multiple exposures can be taken over a 24 hr period (1-60 min each). Using DIG kit labelled ABP cDNA probe, 5 μg of maize genomic DNA was detected in a dot blot (Fig. 4-8).

4.3.2.4 Dot and Southern hybridization analysis of sugar beet genomic DNA

The simplest type of hybridization analysis is carried out using dot blots which are used

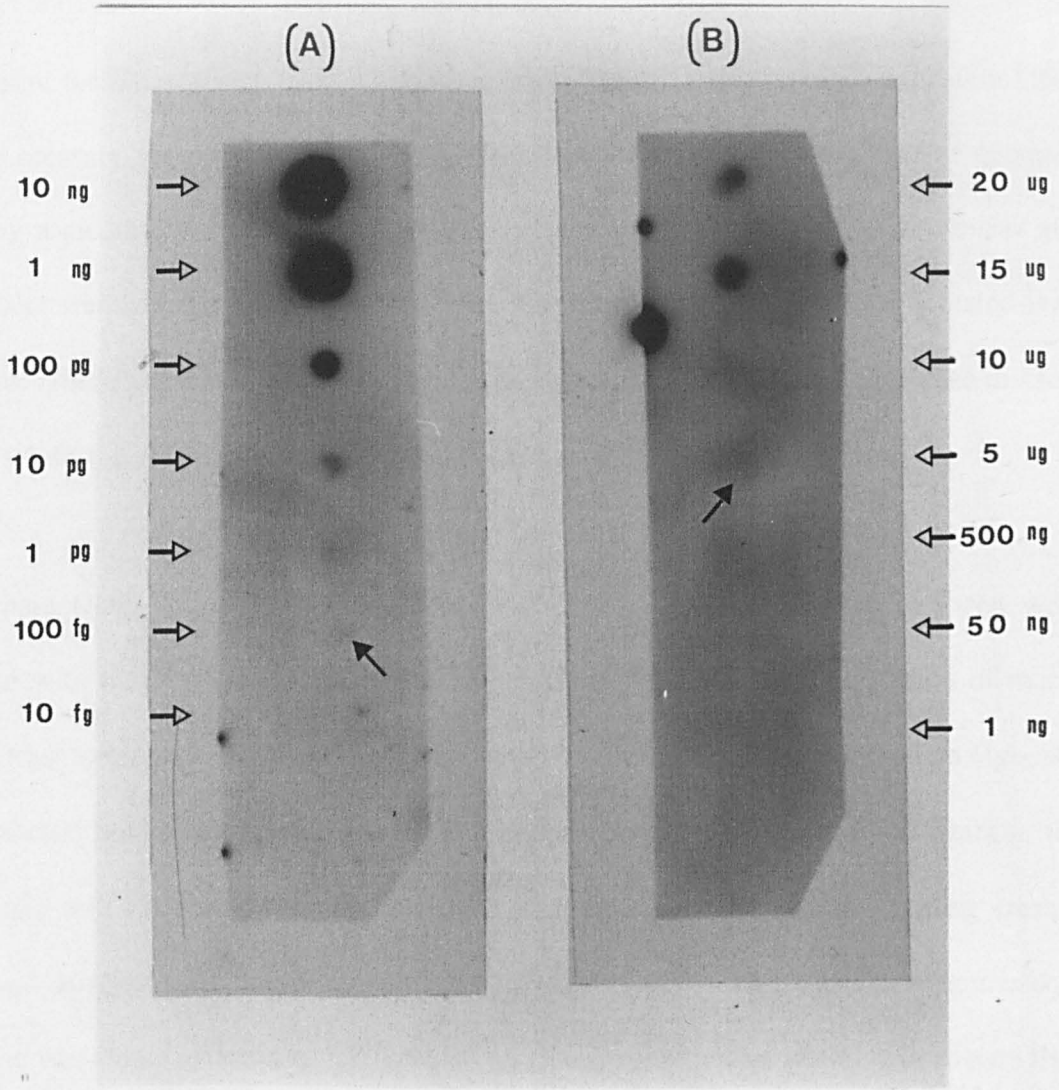


Fig. 4-8 Immuno-chemiluminescent detection of hybridized non-radioactive labelled probes.

A: 0.1 pg of homologous control-DNA1 could be detected when the filter was hybridized with labelled control-DNA2.
B: 5 μg of maize genomic DNA could be detected when the filter was hybridized with labelled maize ABP cDNA.

to measure the abundance of target sequences in the sample. High molecular weight DNA is not necessary for dot blot analysis and it is preferable to reduce the size of genomic DNA by sonication or digestion with restriction enzymes. Genomic DNA from maize and sugar beet were digested with *Pvu*II, *Eco*RI and *Hind*III (Fig. 4-9). When digested with *Eco*RI or *Hind*III, a smear of restriction fragments was found, with several intense discrete bands, while the *Pvu*II resulted an uncomplete digestion.

To prepare DNA for dot blot analysis, both maize and sugar beet genomic DNA were digested with two restriction enzymes, i.e. *Eco*RI and *Hind*III. Series dilutions of maize DNA (30μg to 600pg) and sugar beet DNA (40μg to 800pg) were immobilized on Hybond-N membrane by UV crosslinking on a UV transilluminator. After pre-hybridization, the membrane was incubated with 5 cm^3 hybridization solution containing 120ng freshly denatured labelled ABP cDNA probe for 14 hrs at 42°C. Immuno-chemiluminescent detection was then applied. Fig. 4-10 shows an overnight exposure of the hybridized DNA samples. 30μg, 3μg and 600ng of maize DNA, and 40μg and 4μg sugar beet DNA can be detected. As the result indicated, the sequences in maize and sugar beet are complementary to maize ABP cDNA probe. A disadvantage of the dot blot relative to a Southern blot is that there is less discrimination between correct hybridization and cross-hybridization. The signal seen in a dot blot is the sum of all the hybridizing species within the sample, whereas in a Southern blot a band of strongly-hybridizing material can be picked out of a background smear. Both maize and sugar beet DNA were digested with *Eco*RI and *Hind*III respectively and run on a 0.8% agarose gel. However, a Southern hybridization of non-

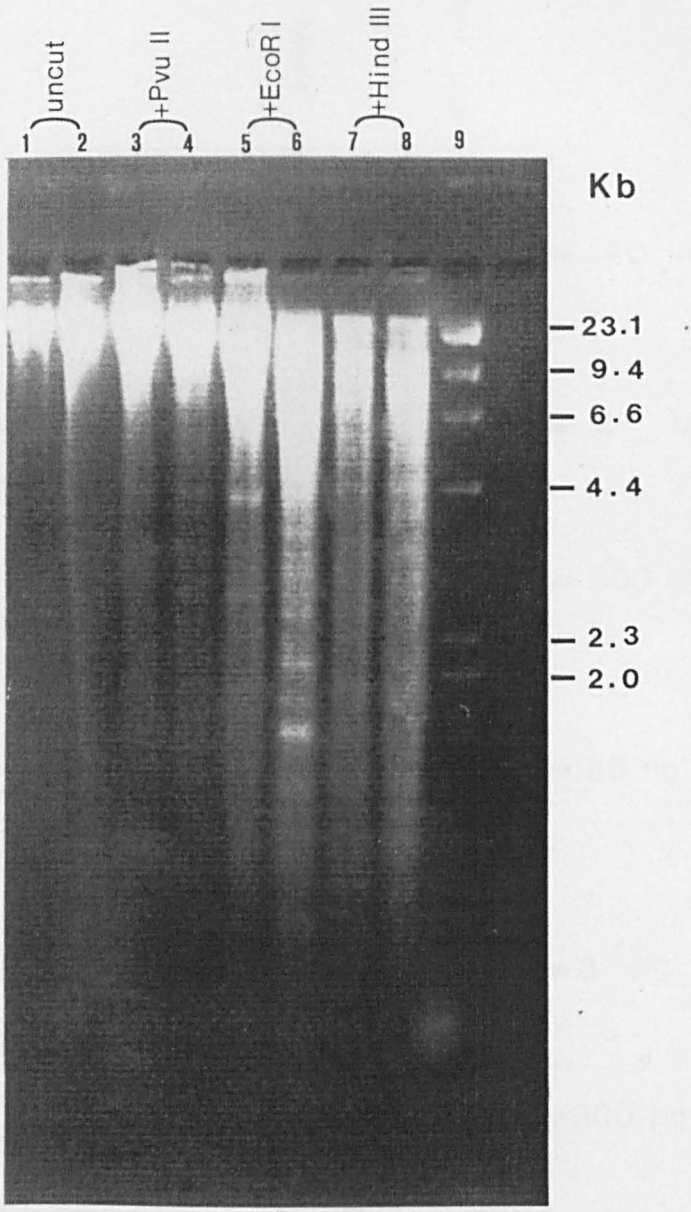


Fig. 4-9 Effective digestion of genomic DNA from maize and sugar beet

DNA samples (4 μg per lane) were digested with the restriction enzymes *Pvu*II, *Eco*RI or *Hind* III.
Lanes 1,3,5,7 maize
Lanes 2,4,6,8 sugar beet
Lane 9 1 μg of Lambda *Hind* III markers

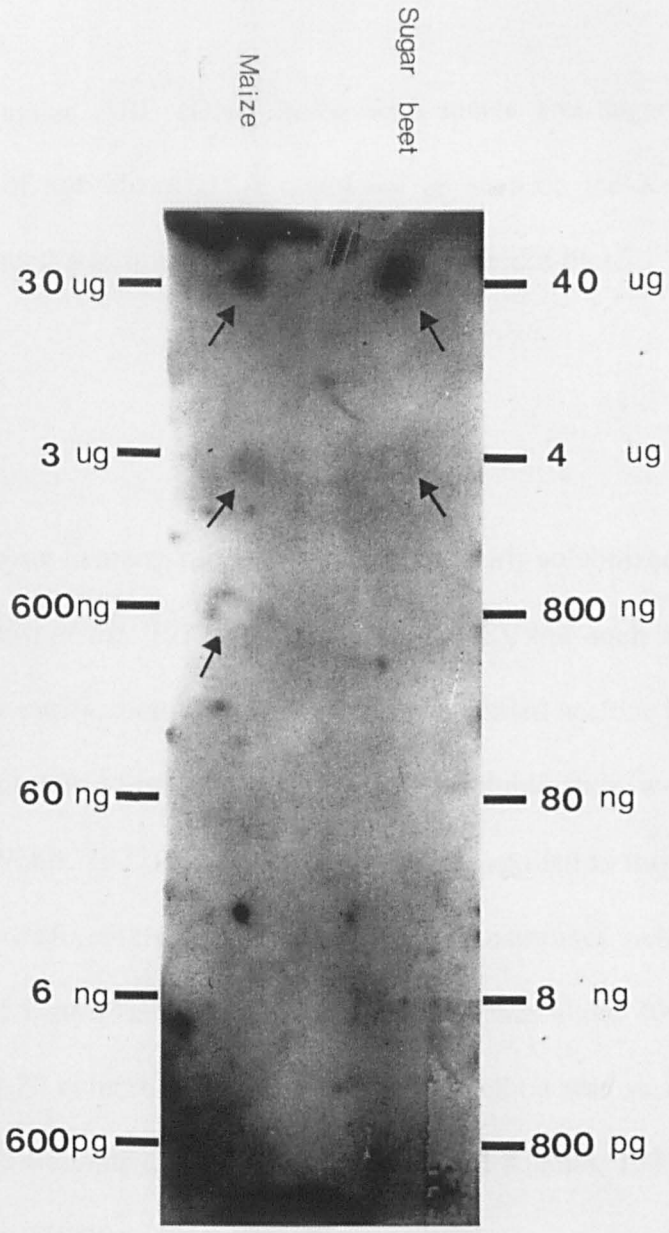


Fig. 4-10 A dot blot hybridization of non-radioactive labelled maize ABP cDNA probe with maize and sugar beet genomic DNA

radioactive labelled maize ABP cDNA probe with maize and sugar beet DNA was unsuccessful. Bands of hybridized DNA could not be seen on the X-ray film and the hybridization background was much higher than that in the dot blot.

4.4 Discussion

The auxin binding protein in maize membrane could be readily solubilized by Triton X-100 (Batt *et al.*, 1976; Ray *et al.*, 1977a; Cross *et al.*, 1978), but such extracts were not amenable to extensive purification. However, use of a modified acetone powder technique enabled the binding protein to be obtained in a buffer-soluble form without recourse to detergent treatment (Venis, 1977). These techniques were applied in this study of ABP in sugar beet. These two different treatments on sugar beet membranes yielded similar levels of ABP in solubilized form (Table 4.5) and the recovery was about 40% as detected by Sephadex-G-25M PD-10 column. The acetone powder method was successfully used by several groups in their attempts to purify ABP (Löbler and Klämbt, 1985a; Shimomura *et al.*, 1986; Napier *et al.*, 1988).

Purification of ABP could be achieved through different combinations of protein purification techniques in general, or the protein can be partially purified through a single step chromatography. A sABP was partially purified in this laboratory, using 2,4-D linked AH-Sepharose 4B affinity column (Bailey *et al.*, 1985). Since Tappeser *et al.* (1981) and Löbler and Klämbt failed in the purification of ABP using an affinity column of 2-hydroxy-

3,5-diiodobenzoic acid-linked Sepharose 4B, selection of an auxin analogue as the affinity absorbent may be critical. α-NAA was successfully used in Shimomura's group (Shimomura *et al.*, 1986) and 2,4-D in this laboratory (Bailey *et al.*, 1985).

In our present work, we followed the method reported by Shimomura *et al.* (1986) involving DEAE-Sephacel (ion-exchange chromatography), NAA-linked AH Sepharose 4B (affinity chromatography)) and Sephadex G-100 (gel filtration). Each of the steps was first used separately on our solubilized fractions containing ABP. Tables 4.7, 4.8 and Fig. 4-3 show that ABP can be partially purified by each single chromatography. However, there was a major problem in that the yield of ABP from any method was not high enough. This became evident when we tried to purify it successively by NAA-linked AH-Sepharose affinity chromatography and DEAE-Sephacel ion-exchange chromatography. Although ABP activity could be detected after ion-exchange chromatography, it was not possible to do so in fractions from the subsequent affinity chromatography. It is not surprising that the low abundant membrane-bound protein like ABP is easily inactivated or lost during purification. This situation is made worse by the fact that ABP abundancy is even lower in sugar beet. For practical reasons, it would be difficult to improve the outcome by increasing the amount of starting material.

Although further purification of ABP from sugar been seedlings seemed to be impractical, its presence in sugar beet encouraged us to isolate the ABP gene in this species and hopefully to identify the regulation of the gene. The first ABP gene, Zm-ER abp1, was

isolated from maize (Hesse *et al.*, 1989; Trillmann *et al.*, 1989; Inohara *et al.*, 1989) and later from *Arabidopsis* (Palme *et al.*, 1992), and the expression of the Zm-ER abp1 gene was shown to be developmentally regulated. However, there is no clear evidence pointing to a receptor function for this gene product (Hesse *et al.*, 1989), mainly because maize cannot be readily transformed, hence approaches commonly used to identify functions of genes, e.g. overproduction of the encoded product or inactivation at any of several levels, cannot yet be addressed routinely in maize. Although many different developmental and hormonal mutants has been identified in maize, the lack of efficient regeneration procedures has prohibited complementation analysis of relevant mutants with the genes of interest (Sheridan, 1982). Effective gene transfer and regeneration methods have been established for sugar beet (*Beta vulgaris* L.) (Lindsey and Gallois, 1990; Grieve, 1990). Thus, this species may enable the study of putative ABP gene and in return the growth and development of this commercially important plant could be regulated through the manipulation of the expression of this gene.

The complete maize ABP cDNA was used as the probe in sugar beet DNA-DNA hybridization. This strategy stands a reasonable chance of success because ABP from distantly related plants, such as maize, cockspur weed, tobacco, or mung beans, share common epitopes that can be detected by immunoblotting using antisera raised against maize ABP (Napier and Venis, 1990; K. Palme and T. Hesse, unpublished results). The non-radioactive DIG DNA labeling and detection method (Boehringer Mannheim) was used in our work. This method, as claimed by the supplier, allows the detection of 0.1 pg of

homologous DNA. Single-copy genes can be detected in 1 μg of mammalian DNA. Compared to radioactive systems it gives faster results with safer handling. Using this technique, hybridization could be detected in 4 μg sugar beet genomic DNA in dot blotting (Fig. 4-10). However, using the same technique, no hybridization band could be detected in Southern blotting with 30 μg loading of genomic DNA. ^{32}P-labeling and detection methods may improve Southern analysis. The use of ^{32}P may in reality provide the large sensitivity margin, though many non-radioactive labels claim equivalent sensitivity to ^{32}P (Mundy *et al.*, 1991). Sugar beet cDNA library and genomic DNA library are available in the laboratory. Screening these libraries may lead to the isolation of a sugar beet ABP gene. Established transformation and regeneration techniques in sugar beet will allow further studies on the function of this protein. It is hoped that sugar beet growth and development could be regulated through ABP gene manipulation.

Chapter 5 General Discussion

5.1 Hormone receptors: function in plant growth and development

Our understanding of the mode of action of plant hormones is lagging behind the level of understanding of hormone action in animals. One of the voids in understanding plant hormone action is our lack of identification and characterization of hormone receptors. Before a hormone can exert its specific physiological and biochemical effects, it must first be recognized by the cell as a molecule belonging to a particular hormonal class. In plants this is true both of naturally-occurring hormones and of those synthetic plant growth regulators that are analogs or mimics of the native molecules. It has long been thought and generally accepted that this function is fulfilled by specific hormone-binding proteins, or receptors (Libbenga *et al.*, 1987; Venis, 1985; Bailey *et al.*, 1985; Elliott *et al.*, 1987, 1988b, 1990). Binding to such receptors would represent the primary level of interaction of a hormone with the cell and interference with or manipulation on this interaction therefore constitutes in principle an attractive target for plant growth regulation.

The first report on plant hormone binding protein was that of Lembi *et al.* (1972) on maize, and the first plant hormone binding protein gene was isolated in 1989 (Inohara *et al.*, 1989; Hesse *et al.*, 1989; Tillmann *et al.*, 1989). During the two decades, the hypothesis of growth substance sensitivity as a factor in determining plant growth and development was formed and advanced in particular by Trewavas (1981). The "sensitivity" concept drew

attention to the importance of the receptor component of these interacting systems.

Changes in sensitivity, i.e. the ability of a tissue to respond to a given concentration of hormone, can be related to the abundance and/or affinity of hormone receptors. Many mechanisms for regulating receptors and biological responsiveness have been reported in animal systems (see e.g. Silbey and Lefkowitz, 1985). Comparable indications are only fragmentary in plants, with no detailed information on possible mechanisms. In tobacco cultures, there is evidence that both membrane-bound and soluble auxin binding proteins are modulated during the growth cycle (Vreugdenhil *et al.*, 1979; Oostrom *et al.*, 1980). NPA binding proteins were studied in *Acer pseudoplatanus* L. cells in this laboratory and they seem to play a vital role in the perception of auxin transport inhibitors and consequently, in regulating IAA accumulation and cell division (Elliott *et al.*, 1988b, 1990).

A simple, but elegant experimental system was developed in Guern's laboratory, relying on the use of antibodies against ABP and the fact that hyperpolarization of isolated protoplasts is an auxin-specific response (Ephritikhine *et al.*, 1987; Shen *et al.*, 1988). Monoclonal antibodies to maize ABP were shown to block this response in tobacco protoplasts (Barbier-Brygoo *et al.*, 1989). Antiserum to a plasma membrane H^+-ATPase blocks auxin-induced hyperpolarization. This work suggests that some ABP is present on the outside of the plasma membrane, and they are tightly coupled to the activity of the H^+-ATPase and are essential for this auxin-induced response. In an auxin-resistant tobacco mutant, both cell division and protoplast hyperpolarization response curves to auxin were

shifted to higher concentrations (Barbier-Brygoo *et al.*, 1990a; Ephritikhine *et al.*, 1987). Conversely, protoplasts from plants transformed with *Agrobacterium rhizogenes* were over 100-fold more sensitive with respect to auxin-induced hyperpolarization than normal protoplasts (Shen *et al.* 1988). Furthermore, titration of the hyperpolarization response with ABP antibodies suggested that mutant protoplasts (less auxin sensitive) had fewer immunoreactive sites at the cell surface than control protoplasts (Barbier-Brygoo *et al.*, 1990b), while with transformed protoplasts this number was increased (Barbier-Brygoo *et al.*, 1990c). Thus, incubation of protoplasts with ABP antibodies shifts the hyperpolarization response curve to higher auxin concentrations, while the addition of purified ABP has the opposite effect (Barbier-Brygoo *et al.*, 1990a,b,c). In other words, it is possible to reproduce mutant or transformed phenotypic sensitivity to auxin by modulating the ABP population at its action site.

These reports have indicated:

(1) The properties of plant hormone receptors are related to plant growth and development. It is thus likely that some mechanism may exist whose function is to regulate the receptor status in plant cells.

(2) The observations are very encouraging in that plant growth and development can probably be regulated by the manipulation of plant hormone receptors, e.g. through genetic engineering.

It is these indications and probabilities that have formed the strategy underpinning our present study. Sugar beet (*Beta vulgaris* L.) was chosen for our work for its agricultural and commercial importance (Elliott *et al.*, 1988a). The work was carried out in two systems. Suspension-cultured sugar beet cells were used for the study of phosphorylation of NPA receptors, whilst sugar beet seedlings were used for the purification of auxin binding proteins and the isolation of gene(s) coding for this protein.

5.2 NPA receptors: characterization and phosphorylative regulation

NPA binding proteins have been widely identified and characterized due to their important role in the regulation of auxin transport (Rubery, 1987). Endogenously synthesised auxin, IAA, must be transported basipetally from its synthesis sites in shoot apices and young leaves to the subapical target tissues. The current hypothesis describing the mechanism of polar auxin transport includes H^+ gradient-driven cytoplasmic auxin accumulation by diffusion of hydrophobic undissociated IAA molecules (Raven, 1975; Rubery and Sheldrake, 1974) and carrier-mediated co-transport of IAA anions and H^+ ions (Hertel, 1983; Lomax *et al.*, 1985; Sabater and Rubery, 1987) and a transmembrane efflux of IAA anions (Sabater and Rubery, 1987) on a carrier preferentially localised at the basal end of cells in the transport pathway (Jacobs and Gilbert, 1983). A group of synthetic compounds, exemplified by NPA, can inhibit polar auxin transport, apparently by blocking the polar efflux step and therefore causing a net IAA accumulation in transporting cells (Rubery, 1987).

NPA binding proteins were intensively studied in this laboratory in two strains of *Acer pseudoplatanus* L. cells (Elliott *et al.*, 1988b, 1990). Suspension cultures of these cells normally need 2,4-D if cell division were to occur. Transferring these "2,4-D requiring (DR)" strain cells into 2,4-D free medium caused reduction in rates of cell division. A "no 2,4-D requiring (NDR)" strain had been adapted to auxin-free medium and had similar culture growth parameters to DR strain cells. The intracellular IAA concentration was 6 times higher in NDR cells than in DR cells. Both strains of cells contained a high affinity membrane-bound NPA binding protein with Kd of 7.5 x 10^{-9} mol dm^{-3}. NDR strain cells always contained a lower concentration of the binding proteins than DR strain cells (pmoles mg^{-1} membrane protein); this lower capacity could be consistent with the maintenance of higher intracellular IAA concentration and growth in the absence of 2,4-D. Studies in DR cells also indicated that NPA binding protein status was also regulated throughout the batch culture cycle (Elliott *et al.*, 1988b, 1990). The number of NPA binding proteins varied during the culture passage. One peak arose from day-0 to day-4 and the other occurred at day-14, leaving a minimum around day-9. Taking both the levels of NPA binding proteins and the levels of intracellular IAA into account, it was apparent that the IAA level tended to increase while the amount of specific binding of NPA decreased. It seems possible that the intracellular IAA levels are intrinsically linked to the status of NPA binding proteins. The intracellular IAA peak appeared to be just within the exponential phase of the batch culture cycle. These studies not only have indicated the importance of NPA binding proteins in the regulation of IAA transport and hence cell division but may also suggest that the properties and the function of NPA binding proteins were controlled by some yet

unidentified mechanism(s). This latter part has been rarely tackled in the field of plant hormone receptor studies. Work reported here has tried to investigate one of the possible mechanisms, i.e. receptor phosphorylation and dephosphorylation.

The presence of a high affinity (Kd 1.71 x 10^{-7} mol dm^{-3}) membrane-bound NPA binding protein was demonstrated in the membrane preparations from sugar beet (*Beta vulgaris* L.) cell suspension culture. The binding protein was characterized in its pH optimum for *in vitro* binding assay, reversibility of the binding reaction, displacement by chemicals, as shown in section 3.3. This experimental system was further employed in our preliminary attempt to study the possible role of phosphorylation and dephosphorylation in the regulation of this binding protein.

Using cells harvested 5-10 days after subculture, Mg^{2+}ATP was shown to increase NPA binding. This activation was tested reproducibly, and the binding was increased by 30% (Fig. 3-7). The effect of acid phosphatase, on the other hand, was shown to inactivate this binding by 33% (Fig. 3-8). This inactivation was concentration dependent. Moreover, it was noticed that the effect of Mg^{2+}ATP and acid phosphatase on NPA binding was culture stage dependent. Cells from day 1 (representing the lag phase), day 8 (representing the linear phase) and day 14 (representing the stationary phase) were tested. Although a considerable effect could be detected when using cells from day 8, the same treatment did not alter the binding when using cells from day 1 or day 14 (Fig. 3-10). All these observations, together with the evidence of the presence of endogenous phosphatase activity in these cells,

strongly highlight the possible involvement of phosphorylation and dephosphorylation mechanism *in vivo* in the regulation of the activity of NPA binding proteins.

Phosphatases are also known to inactivate hormone binding to steroid receptors. In some cases they seem to be fully responsible for the rapid inactivation of steroid receptors at elevated temperatures (Traish *et al.*, 1981; Auricchio *et al.*, 1981a,b; MacDonald *et al.*, 1982, 1983; Abou-Issa *et al.*, 1982; Munck *et al.*, 1972; Barnett *et al.*, 1980; Nielsen *et al.*, 1977a,b; Sando *et al.*, 1979a). Binding to these receptors can be restored by addition of a reducing agent, such as DTT, and/or by activating protein kinases by adding Ca^{2+} or Mg^{2+}ATP (Sando *et al.*, 1979a,b; Auricchio *et al.*, 1981a,b, 1982; Migliaccio and Auricchio, 1981; Migliaccio *et al.*, 1982, 1984). Additional, ATP might act by phosphorylating a regulatory site of the protein, as is known in animals for steroid hormone receptors (Sloman and Bell, 1976). This has been proposed as a universal regulatory mechanism for membrane receptors (Sibley *et al.*, 1987). Our work suggests that such phosphorylation and dephosphorylation mechanisms of receptor regulation are not restricted to the animal kingdom and it could also be one of the major mechanisms in the regulation of the properties of plant hormone receptors. It can be suspected that receptors for other plant growth regulators may also be modulated by this mechanism.

5.3 Auxin receptors: purification and gene isolation

A crucial step in auxin receptor work is to purify the proteins so that amino acid

sequencing and DNA sequencing would become possible. Ideally, the work described above on the NPA receptor could be developed to lead to its purification, so that the protein structure and the binding mechanisms could be investigated. It is possible that this may also help to understand the mechanisms of its regulation by phosphorylation and dephosphorylation. However, previous work in this laboratory and others (e.g. Morris, personal communication) has proved frustrating. Recovery of NPA binding proteins after solubilization was lower than 5% in *Acer pseudoplatanus* L. cells. The success in solubilization and purification of auxin binding proteins and the success in the isolation of the gene encoding this protein (see 1.4.1) suggest a more promising system for further studies of plant hormone receptors. Protein structure study and its gene analysis will help in our understanding of its roles in auxin action and may offer ways for us to regulate the receptor function through protein modification or to regulate its expression through gene manipulation.

The presence of specific auxin binding was demonstrated by Scatchard analysis (Scatchard, 1949) in sugar beet seedlings. The Kd value was shown to be 2.15 x 10^{-6} mol dm^{-3}. Two of the most commonly used methods in ABP solubilization, i.e. Triton X-100 and acetone methods, were attempted in solubilizing this protein from its membrane-associated status in sugar beet. Both methods proved to be successful and about 40% of the binding activity could be recovered after solubilization (Table 4.5). In view of its simplicity, the acetone powder method was routinely used.

The ABP solubilized from maize shoot membranes was successfully purified by a series of chromatographics on DEAE-Sephacyl, α-NAA-AH-Sepharose 4B and Sephadex G-200 by Shimomura *et al.* (1986). Their protocols were followed in our present work. ABP could be partially purified when using any of these chromatographic methods, although the recovery was very low (Table 4.7, 4.8, Fig. 4.3). When trying to further purify it by these chromatographies in succession, binding activity could not be detected after the second step of purification. Several factors could be responsible for this observation, i.e. the plant species, the amount of starting material and ABP inactivation or loss during the purification procedure. Compared to maize, the abundance of ABP in sugar beet is much lower. In terms of pmoles mg^{-1} membrane protein, the amount of ABP in sugar beet is only 10% of that in maize (Shimomura *et al.*, 1986). This level of ABP is comparable to many other plant materials (Kaur and Kapoor, 1989: Vreugdenhil *et al.*, 1979; Jacobs and Hertel, 1978; Patel *et al.*, 1986; Cross and Briggs, 1978). For the purification of proteins with low abundancy, the amount of the starting material was critically important. To obtain purified ABP reproducibly, 3.4 Kg or more of maize shoot was used by Shimomura *et al.*(1986). It can be expected that much more starting material will be necessary for ABP purification in sugar beet. As much material as possible within the experimentally managable limit was used in our work, but poor recovery coupled with low starting levels prevented purification.

Although ABP could not be further purified in our work, its existence had encouraged us in the search for the gene coding this protein. This was made possible by the work of Hesse *et al.* (1989), Tillmann *et al.* (1989) and Inohara *et al.* (1989). These groups reported

the molecular cloning and structural analysis of the gene coding for ABP in maize. Later, Palme *et al.* (1992) reported ABP gene analysis in *Arabidopsis*. A complete maize ABP cDNA was kindly provided by Dr. C.M. Lazarus of Bristol University and was used as the probe in our DNA hybridization in sugar beet. Using a non-radioactive labelling and detection technique, this probe was shown to hybridize to sugar beet genomic DNA in dot blotting. Although hybridization could not be detected in Southern blotting, the result from dot blotting is promising. Further work may require the use of ^{32}P-labelled probe to improve the detection sensitivity in Southern analysis. Screening of sugar beet cDNA and genomic DNA libraries, which are available, may lead to the isolation of ABP gene in sugar beet. This work is underway in this laboratory.

5.4. Summary: towards plant growth regulation through hormone receptor manipulation

Hormones exert physiological and biochemical effects that are specific to their particular class. The hormone molecules must be recognized in the cell and distinguished in some way from other compounds. It has long been suspected and generally agreed that specific hormone binding proteins (receptors) may fulfil this function. The binding of the hormone is assumed to activate the receptor in a manner such as to transduce the hormone signal. With the increasing use of techniques of biochemistry and molecular biology, efforts to characterize hormone receptors in plants and unravel mechanism(s) of signal transduction are intensifying (Venis, 1985; Venis and Napier, 1991) and recently, the plant hormone

receptor gene (ABP gene) was first isolated (Hesse *et al.*, 1989; Inohara *et al.*, 1989).

The role of plant hormone receptors in the regulation of hormone function has been intensively investigated in this laboratory in *Acer pseudoplatanus* L. cells (Elliott *et al.*, 1987, 1988b, 1990) and in tobacco cells (Bailey *et al.*, 1985). These studies and those from other laboratories (Napier and Venis, 1991; Van der Linde *et al.*, 1984; Jacobsen and Hajek, 1985; Shen *et al.*, 1988; Ephrithikhine *et al.*, 1987; Barbier-Brygoo *et al.*, 1990a,b,c; Hick *et al.*, 1989) have led to our current view that plant hormone receptors could be one of the determinants of plant response to its hormones. However, as the first stage of signal recognition and perception, the regulation of the receptors themselves, though critically important, has attracted little attention. In our present work, regulation of NPA binding protein through phosphorylation and dephosphorylation and regulation of ABP through gene manipulation have been attempted.

The presence of a high affinity NPA binding protein has been demonstrated in sugar beet cell suspension culture. Its activity was shown to be activated by Mg^{2+}ATP and inactivated by acid phosphatase in the *in vitro* binding assay. This effect of activation and inactivation was strictly dependent on the culture stage of the cells. A high level of endogenous phosphatase activity was found in the cells, which suggested that a phosphatase may play a role in the inactivation or regulation of this binding protein *in vivo*. Our work has indicated that phosphorylation and dephosphorylation of receptors could be one of the mechanisms for the regulation of receptor function.

Further studies on membrane-bound auxin receptors will be aimed at analysis of receptor structure and regulation. Although its purification was difficult mainly due to the low abundance of this protein, DNA hybridization analysis has indicated the presence of the gene sequence coding this protein in sugar beet. The isolation of this gene and the manipulation of its expression, e.g. overproduction of the encoded protein by using transformation techniques, may make it possible that the effect of auxin on plant growth and deveplopment can be regulated through the manipulation of the expression of its receptors.

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